Investigating the Hepatitis C Virus (HCV) RNA Translation Modulation by Non-Structural Protein 5A (NS5A)

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By

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ABSTRACT

Hepatitis C virus (HCV) non-structural protein NS5A is a multifunctional protein and despite lacking enzymatic activity has critical roles in viral replication and assembly. The role of NS5A in HCV RNA translation has not been well studied. In an attempt to better understand the role of HCV NS5A in RNA translation, our previous work showed that HCV-1b NS5A downregulates viral RNA translation by binding to the poly(U/UC) region in the 3'UTR. All three domains are capable of individually downregulating translation, albeit with a lesser effect than the full-length wild-type NS5A.

There are multiple HCV genotypes and NS5A from different genotypes may or may not carry out the same function. Therefore, to determine whether the role of NS5A is conserved in other genotypes, we studied the effect of HCV-2a NS5A on monocistronic HCV-2a RNA reporters and replication defective genomic RNA with or without poly(U/UC) region at the 3'UTR. We found that although HCV-2a NS5A also downregulates viral translation, it does not require the poly(U/UC) region in 3'UTR. The translation downregulation by HCV-2a NS5A was predominantly mediated by domain I. Our results elucidated that HCV-2a NS5A modulates viral translation through a mechanism different from HCV-1b NS5A.

NS5A is a phospho-protein and exists as hypo- and hyper-phosphorylated NS5A. The hyperphosphorylation of NS5A is mediated through the phosphorylation of the conserved serine residues cluster in the low complexity sequence LCS I. The serine residues are S222, S225, S229, S232, S235 and S238. Phosphorylation on these serine residues has been found to be important for HCV replication and viral assembly. To further understand the significance of NS5A hyperphosphorylation on HCV life cycle, we investigated the role of HCV-1b NS5A hyperphosphorylation on translation by analyzing the effects of phospho-ablative and phospho-mimetic mutants of the six serine residues on HCV-1b genomic RNA translation. We showed that phosphorylation of S222, S225, S235 is not involved in translation downregulation by NS5A. In contrast, alanine mutations at S229 or S238 can no longer downregulate translation, whereas S229D or S238D mutations have no effect. Interestingly, S232D, but not S232A, abrogates translation downregulation by NS5A.

NS5A exists as a dimer and its dimerization is important for regulating its function. Therefore, we studied the effect of phospho-mutants of S229, S232, and S238 on dimerization in a protein-protein interaction assay and showed that phospho-mimetic S229D or S238D mutations enhance NS5A dimerization, whereas the phospho-ablative mutations of them have no effect. In contrast, neither phospho-ablative nor phospho-mimetic mutations of S232 affect dimerization. In conclusion, these results indicated that phosphorylation of NS5A at S229, S232, and S238 is involved in viral translation regulation and NS5A dimerization.

In summary, these findings suggest that NS5A downregulates the translation of HCV RNA however, the mechanism may differ within the genotypes. In addition, hyperphosphorylation of NS5A is involved in regulation of HCV translation and NS5A dimerization. These results aid in the understanding the mechanism involved in regulation of viral translation by NS5A and may help in the development of pan-genotypic novel antiviral targets.

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Lastly, I would like to thank my parents and my brother for their constant love and support. Everything I have achieved in my life, I owe to them.

DEDICATION

This thesis is dedicated to my mother Mrs. Manju Subba and

my father Mr. Keshar Kandangwa,

For their selfless love and eternal support.

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LIST OF ABBREVIATION

HCV	-	Hepatitis C virus
GT	-	Genotype
UTR	-	Untranslated regions
NS5A	-	Non-structural protein 5A
RdRP	-	RNA dependent RNA polymerase
SVR	-	Sustained virologic response
Peg-IFN	-	Pegylated interferon
DAA	-	Direct Acting Antivirals
NI	-	Nucleotide inhibitors
NNI	-	Non-nucleotide inhibitors
RAV	-	Resistant associated variants
RAS	-	Resistance associated substitution
ER	-	Endoplasmic reticulum
IRES	-	Internal ribosomal entry site
AH	-	Amphipathic α-helix
DI	-	Domain I
DII	-	Domain II
DIII	-	Domain III
LCS	-	Low-complexity sequence
CypA	-	Cyclophilin A

NSP1L1	-	Nucleosome assembly protein 1-like
Bin 1	-	Bridging integrator 1
VAP-A	-	Vesicle-associated membrane protein-associated protein A alpha
PI4KIIIα	-	Phosphatidylinositol 4-kinase type III
PI4P	-	Phosphatidylinositol 4-phosphate
CKIα	-	Casein kinase Iα
CAMK2	-	Calmodulin-dependent kinase II

1.0 LITERATURE REVIEW

1.1 Hepatitis C virus

1.1.1 Identification and Molecular characteristics

Hepatitis C virus (HCV) is an enveloped, positive-sense, single-stranded RNA virus belonging to the *Hepacivirus* genus within the *Flaviviridae* family. On the basis of phylogenetic and sequence analyses of the whole HCV genome, the HCV isolates are classified into seven major HCV genotypes (1-7) and 67 confirmed and 20 provisional subtypes (Smith et al., 2014b). There is a difference of 30-35% nucleotides within different genotypes and of <15% within subtypes (Smith et al., 2014b).

The HCV genome is 9.6 kb in size and comprises a single open reading frame (ORF) flanked with 5' and 3' untranslated regions (UTRs) (Fig. 1.1.1). The UTRs are involved in the modulation of viral translation and replication (Shi and Lai, 2006). The ORF is translated into as single polyprotein of approximately 3000 amino acids which is cleaved co- and post-translationally by viral and host proteases into three structural and seven non-structural proteins. The structural proteins are produced at the N-terminus of the polyprotein and include the core, E1, and E2. The non-structural (NS) proteins include p7, NS2, NS3, NS4A, NS4B, NS5A and NS5B. Among the NS proteins, NS2 is a protease required for virion assembly (Jirasko et al., 2008; Jirasko et al., 2010). NS3 is a bifunctional protein. The N-terminal region of NS3 is a serine protease responsible for cleaving individual proteins from NS3-NS5B polyprotein while the C-terminal portion holds helicase/nucleotide triphosphatase (NTPase) activity (De Francesco and Steinkuhler, 2000). NS4A is a cofactor for the NS3 serine protease (De Francesco and Steinkuhler, 2000). NS4B serves as the scaffold for the HCV replication complex (RC) by inducing the formation of the membranous web (MW) in the cell (Egger et al., 2002). NS5A is a phosphoprotein that plays key roles in HCV RNA replication and viral assembly processes (Macdonald and Harris, 2004; Ross-Thriepland and Harris, 2015). NS5B is the viral RNA-dependent RNA polymerase (RdRP) (Behrens et al., 1996). The HCV RdRP lacks proofreading function and is error-prone. The RdRP generates one mutation per 10^{-3}–10^{-5} nucleotides resulting in approximately one or more nucleotide substitution per replicated genome

(Shimakami et al., 2009). The high replication error rate and virion production (10^{12} particles per day) cause high diversity in the sequences of virus in one patient and the pool of mutated viruses is termed as quasispecies (Neumann et al., 1998).

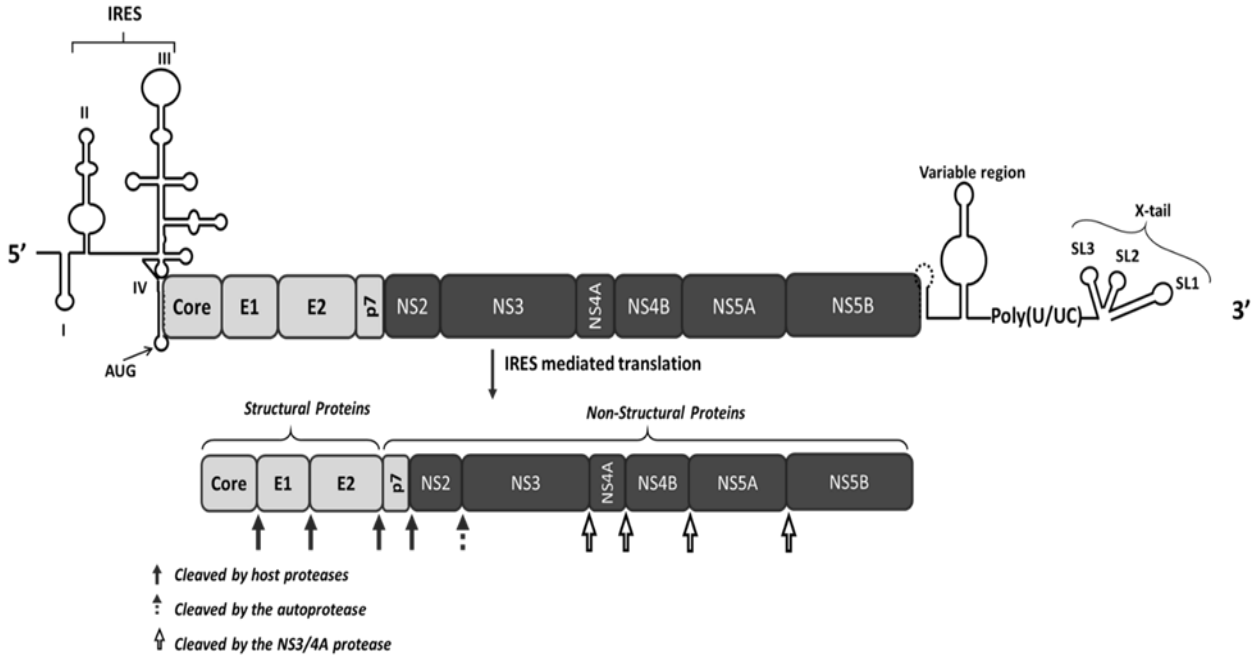

Figure 1.1.1: **Schematic representation of HCV genome and its polyprotein processing.** The HCV genome is approximately 9.6 kb and is flanked with highly structured 5' and 3' UTR. The HCV genome is translated in an IRES dependent manner to yield a polyprotein precursor that is processed by host and viral proteases into three structural proteins (Core, E1 and E2) and seven non-structural proteins (p7, NS2, NS3, NS4A, NS4B, NS5A and NS5B).

1.1.2 Epidemiology

Hepatitis C is a non-hepatitis A and non-hepatitis B liver disease caused by the hepatitis C virus (HCV). HCV was first identified in 1989 and since then laboratory testing aimed at detecting HCV infection has been available. At present, an estimate of 71 million people are living with HCV world-wide (WHO Hepatitis C Fact Sheet, July 2019. https://www.who.int/news-room/fact-sheets/detail/hepatitis-c). HCV is classified into 7 genotypes and their prevalence varies by geographical region. HCV GT1 is the most prevalent throughout the world and comprises 46% of all HCV cases (Messina et al., 2015). HCV GT3 is the second most prevalent and comprises 30% of total HCV cases and is common in South Asia, Australasia and some European countries (Gower et al., 2014; Messina et al., 2015). The GT2 and 4 are the next most

common representing 13% of HCV infections (Gower et al., 2014). GT2 is more prevalent in Asia and West Africa while GT4 cases are more prevalent in Central and Eastern sub-Saharan Africa, North Africa and the Middle East (Gower et al., 2014; Messina et al., 2015). HCV GT5, 6 and 7 comprise of less than 1% of total infection (Messina et al., 2015) with GT7 being the least common, as only a small number of cases has been reported (Murphy et al., 2015). Distribution of GT5 is common in South Africa, GT 6 in East and South Asia (Messina et al., 2015) and GT7 in Democratic Republic Congo (Murphy et al., 2015).

1.1.3 Clinical characteristics

HCV incubation period ranges from 15 to 150 days and develops into the acute stage of infection. At acute stage, 70- 90% of infected people are asymptomatic, while 10-30% of the patients manifest non-specific symptoms such as appetite loss, flu-like signs or musculoskeletal pain (Lauer and Walker, 2001). In 80% of cases, infection develops into a chronic HCV infection while spontaneous HCV elimination may occur in 20% of cases. 25% of chronic HCV infections are mild with no cirrhosis and the proper functioning aminotransferases while the rest develop chronic infection that results in fibrosis (15-30%), cirrhosis (20-33%) or hepatocellular carcinoma (approx. 2%) over a period of 20 years or more (Lauer and Walker, 2001; Zając et al., 2019). Hepatocellular carcinoma can occur without cirrhosis but is rare.

1.1.4 Transmission

HCV is a blood borne virus and primarily transmitted through percutaneous contact with infected blood. Prior to its discovery in 1989, a large population got infected through contaminated blood transfusions. Although blood testing has greatly reduced the risk of HCV transmission still new infections persist. At present, HCV infections occur in among marginalized populations such as people who inject drugs (PWIDs), imprisoned individuals and men who have sex with men and also the people in developing countries (Thomas, 2013). These individuals at risk are mostly disengaged from medical care with limited access to HCV screening and treatment. In the meantime, they continue to infect others and contribute to the

ongoing epidemic. Access to sterile medical equipment and procedures also remain a major cause of new HCV infections in developing countries (Thursz and Fontanet, 2014). Transmission through piercing or tattooing are also possible through the use of unsterile and HCV contaminated equipment (Westbrook and Dusheiko, 2014). Another factor for emergence of new cases of HCV is due to the lack of a preventive HCV vaccine.

1.1.5 Diagnosis

Acute and chronic HCV infection is diagnosed based on the detection of HCV RNA, HCV antibodies and/or increased activity of alanine aminotransferase (ALT). HCV RNA can be detected 1-3 weeks after infection by a sensitive molecular method with lower limit of detection of <15 international units [IU]/ml. HCV antibodies can be detected after 4-10 weeks by enzyme immunoassay (EIA). However, in early acute hepatitis C and in profoundly immunosuppressed patients the EIA results may be negative, in such cases the presence of HCV RNA is a sign of infection.

A patient is diagnosed as acute hepatitis when anti-HCV antibodies is positive, ALT is 10 times higher than the upper limit of normal level and shows jaundice, with the absence of a history of chronic liver disease or other causes of acute hepatitis. During the acute phase, a brief period of undetectable HCV RNA may occur.

A patient is diagnosed with chronic hepatitis C when the detection of both HCV antibodies and HCV RNA are positive. In the case of newly acquired HCV infection, the diagnosis of chronic hepatitis C can be performed four to six months post-infection as spontaneous viral clearance is rare beyond this time period (EASL, 2014).

1.1.6 Treatment

Traditionally, HCV was treated using interferon alpha only and had very low sustained virologic response (SVR). SVR is defined as a lack of HCV RNA detection 12-24 week following treatment. Since 2001, HCV treatment involved the combination of pegylated interferon (Peg-

IFN) and weight-based doses of ribavirin. This therapy increased the overall rate of SVR from less than 20% to greater than 60%. The rate of SVR was 40%-60% in "difficult-to-treat" genotypes 1 and 4 with 48 week treatment and 70%-90% of "easy-to-treat" genotypes 2 and 3 patients with 24 week treatment (Tsubota et al., 2011). The regimen included a weekly subcutaneous injection of pegylated interferon alpha along with ribavirin tablets given two times a day. The therapy has several adverse effects such as flu-like symptoms, vomiting, nausea, depression, mood swings, central nervous system disturbances and anemia (Sulkowski et al., 2011).

1.1.6.1 Direct Acting Antivirals (DAAs)

Over the past few years, treatment options for HCV have grown exponentially. Currently, as standard-of-care treatment for HCV infection, direct-acting antiviral (DAAs) are used. DAAs are drugs that target HCV non-structural proteins involved in viral replication and infection. Four classes of DAAs exist categorized according to the protein they inhibit i.e. NS3/4 protease, NS5A, and NS5B polymerases (nucleoside and non-nucleoside).

NS3/4A protease inhibitors (-previr): The DAAs that fall in this class normally have –previr as suffix for example: glecaprevir, grazoprevir, paritaprevir, simeprevir and voxilaprevir. Boceprevir and telaprevir (withdrawn from the market) were NS3/4A protease inhibitors and were the first generation DAA to be approved by FDA and EMEA for the treatment of HCV GT1 (EASL, 2014). After two years, the second-generation DAA simeprevir was released. Soon after third generation DAA Glecaprevir, grazoprevir, paritaprevir and voxilaprevir were released. These inhibitors function by inhibiting the enzymatic activity of NS3/4A polymerase. This inhibition prevents the cleavage of HCV polyprotein, thus affecting different downstream stages of viral life cycle mediated by the viral proteins (Grakoui et al., 1993).

NS5A inhibitors: The DAAs of this class have –asvir as the suffix: declatasvir, elbasvir, ledipasvir, ombitasvir, pibrentasvir, velpatasvir. Daclatasvir, the first discovered NS5A inhibitor. It was approved by EMEA in 2014 and by FDA and Canada in 2015. The mode of action of the NS5A inhibitor is not fully understood; however, based on mathematical modeling declatavir is

predicted to block viral replication and virion assembly/secretion (Guedj et al., 2013). The NS5A inhibitors exhibit higher potency than NS3/4A and NS5B inhibitor; however, they are slower at inhibiting viral RNA synthesis (McGivern et al., 2014).

NS5B RNA dependent RNA polymerase inhibitors (-buvir): The DAAs of this class have –buvir as the suffix: sofosbuvir and dasabuvir. NS5B inhibitors are divided further as the class of nucleotide inhibitors (NIs) and the class of non-nucleotide inhibitors (NNIs).

The NIs work by targeting the active site of NS5B polymerase to get incorporated in the nascent viral RNA and prevent further incorporation of nucleotides. This leads to premature chain termination and consequently interrupts viral replication (Eltahla et al., 2015). Sofosbuvir is a pro-drug, licensed in 2013 and belongs to the class of nucleotide inhibitors (Gerber et al., 2013). Sofosbuvir targets the highly conserved active site of NS5B and thus demonstrates antiviral activity across all HCV genotypes. Therefore, it is a pan-genotype DAA and exhibits a high genetic barrier to the development of drug resistance (Eltahla et al., 2015; Stedman, 2014).

Unlike NIs that compete with uridine triphosphate to bind to the NS5B, the NNIs are non-competitive. NNIs bind to one of the four allosteric sites on NS5B, inhibiting the functional conformational change of NS5B required for its RNA dependent RNA polymerase (RdRp) activity (Caillet-Saguy et al., 2011; Davis et al., 2015). In addition, compared to NI, NNIs have lower genetic barrier to development of drug resistance. Dasabuvir is a non-nucleotide inhibitor licensed in 2013 (Gerber et al., 2013).

1.1.6.2 Limitations of DAA

The use of DAAs has significantly improved the sustained virologic response (SVR) rate of HCV treatment. However, in some cases the treatment fails. One of the reasons for DAA treatment failure is the emergence of drug resistant associated variants (RAVs) of HCV. RAVs have specific amino acid substitutions in the viral protein/s targeted by DAAs thus reducing viral sensitivity to the drug. Existence of RAVs in patients may occur naturally or could be selected during the DAA treatment. The likelihood of RAV to be selected and outgrow the native viral population during DAA regimen depends on the DAA's genetic barrier to resistance, level of

drug exposure and the viral fitness of the RAV (Lontok et al., 2015) . In both cases, the prevalence and resistance can vary within HCV genotype and subtype and is also affected by efficacy of specific DAAs. The genetic barrier to resistance is lower in NS3/4A, NS5A and non-nucleotide NS5B inhibitors compared to nucleotide NS5B inhibitors. Since NS5A inhibitors have higher potency, resistance associated substitutions in the NS5A region is more important from clinical point of view (Zeuzem et al., 2017). In addition, NS5A RAV can persist for over two-years post-treatment (Paolucci et al., 2015) while those of NS3/4A and NS5B are transient and disappear in few month post-treatment (Buti and Esteban, 2016; Svarovskaia et al., 2014; Wyles et al., 2018). Therefore, to lower the risk of selecting RAVs and decrease the cases of treatment failure and relapse, a combination of two or three DAAs with different targets and mechanism of action has been applied as standard care of practice (Shah et al., 2018).

Another limitation on use of DAA to treat HCV is the case of HCV/ HBV co-infection. US Food and Drug Administration (FDA) has issued warning on the potential risk of HBV reactivation during the therapy or after the clearance of HCV (Bersoff-Matcha et al., 2017). As of now, status of HBV infection in the HCV/HBV co-infected patient is checked prior initiating any kind of treatment (Ma and Feld, 2018).

Discovery of DAAs for the treatment of HCV is one of the biggest achievements in the field of HCV. DAAs were initially released in 2011 and as of now have replaced the old interferon regimens due to its high efficacy, safety and tolerability. So far, no serious side-effects associated with DAAs have been reported. However, it is still too early to determine their long-term effect and thus the patients treated with DAAs follow up study needs to be done in future.

1.1.7 HCV life cycle

The HCV viral particle consists of nucleocapsid formed through the interaction of HCV RNA genome with core protein and enveloped with a lipid bilayer with two viral envelope glycoproteins E1 and E2 embedded on it (Bartenschlager et al., 2011). HCV only infects humans and chimpanzees. The virus particle circulates through the bloodstream and infects the liver.

On the hepatocytes, first HCV interacts/ binds with several cell surface receptors (mainly CD81, SR-B1, CLDN1, OCLN, EGFR, EphA2, NPC1L1) and thereby docking itself onto the surface of the cell (Lindenbach and Rice, 2013). Receptor- bound HCV internalizes into the cell via clathrin-mediated endocytosis (Blanchard et al., 2006). After entry, the HCV particle undergoes pH-dependent membrane fusion within the endosome and releases the viral RNA into the cytoplasm (Meertens et al., 2006). In the rough endoplasmic reticulum (ER) the positive-stranded RNA genome is directly translated in a cap independent manner via the IRES and generates a single polyprotein precursor, which is further processed into mature proteins: three structural and seven non-structural proteins. The structural proteins are dispensable for viral replication while the non-structural proteins have been shown to assist in the assembly of the virus, which is the main function of the structural proteins. After polyprotein processing, replication of the viral genome takes place in a specific membranous web (MW) of the endoplasmic reticulum. The first step of replication is formation of negative-strand RNA genome, which serves as the template for positive-strand RNA replication. This replication is catalyzed by the viral NS5B RNA dependent RNA polymerase (RdRp). The progeny positive-strand RNA genomes are either used for translation giving rise to more viral protein, used for generation of more negative-strand intermediate/ template, or trafficked to the assembly sites to be packed into viral particles. The virion assembly takes place in an ER-derived compartment and viral particles exit cell via Golgi –dependent secretory pathway (Paul et al., 2014). Outside the cell, HCV viral particle exists as a lipo-viro-particle (LVP) with an extremely low buoyant density (Syed et al., 2017).

1.1.8 The functional RNA elements

The HCV 5'UTR is a highly structured nucleotide region containing four stem-loops (I-IV) and is highly conserved amongst different viral strains (Bukh et al., 1992). The first two stem loops are essential for replication (Friebe et al., 2001). The stem loops II-IV (nucleotides 40-341) with a few nucleotides from the core-binding region form a highly structured segment, the internal ribosomal entry site (IRES) (Tsukiyama-Kohara et al., 1992; Wang et al., 1993). The stem loops II and III contain the structural elements crucial for translation initiation and stem loop IV

contains the initiation codon (Hoffman and Liu, 2011). The HCV IRES facilitates the cap-independent translation of the viral RNA by the direct recruitment of HCV translation machinery factors: 40S ribosomal subunit, eukaryotic initiation factor (eIF2)/guanosine triphosphate/Met-transfer RNA complex and eIF3 to initiate translation (Pestova et al., 1998).

The HCV 3'UTR is a unique non-poly(A) tail with a tripartite structure comprising of a short genotype-specific variable region, a tract of variable length comprising solely pyrimidine residues (poly(U/UC)) and a conserved 98-nucleotide sequence, known as the X-tail region (Kolykhalov et al., 1996). The variable region is structured with two stem-loops but is poorly conserved and dispensable for RNA replication (Friebe and Bartenschlager, 2002; Yi and Lemon, 2003a). The poly(U/UC) tract is a unstructured region of variable length and is comprised of stretches of uridines and cytidines with a homouridine core (Friebe and Bartenschlager, 2002). The poly(U/UC) region is essential for replication (Friebe and Bartenschlager, 2002; Yi and Lemon, 2003a) and also plays a role in translation modulation (Hoffman et al., 2015a; Hoffman and Liu, 2011). The X-tail is a highly conserved and structured region with three stem-loops structures: SL1, SL2 and SL3 (Kolykhalov et al., 1996). The composition and integrity of the three stem-loops are critical for replication as even the mutation/s maintaining the structure of the loops have negative impact on replication (Friebe and Bartenschlager, 2002; Yi and Lemon, 2003b).

The 3' UTR is essential for viral replication as it serves as the initiation site for the synthesis of negative strand however its exact role in translation has not been very well elucidated. It has been reported that all three regions of 3'UTR contribute to RNA translation enhancement through a unclear mechanism (Song et al., 2006). Possible mechanism includes correct translation termination possibly mediated through 5' and 3' UTR communication, recruitment of host proteins that could interact with 5'UTR or translation machinery at 5'UTR and ribosome recycling (Bai et al., 2013; Hoffman and Liu, 2011).

1.1.9 NS5A

NS5A is an enigmatic non-structural protein of HCV without any apparent enzymatic activity, however, critical for HCV RNA replication and virion assembly (Macdonald and Harris, 2004; Ross-Thriepland and Harris, 2015).

NS5A consists of an N-terminal amphipathic α-helix (AH) and three domains (DI, DII & DIII) separated by low-complexity sequences (LCS) LCSI and LCSII (Fig. 1.1.9). The AH is associated with anchoring of NS5A within the ER membrane for recruitment to lipid droplets (Paul et al., 2014; Penin et al., 2004). Domain I (DI) is a highly conserved region containing zinc-binding and RNA-binding motifs and plays a critical role in HCV RNA replication (Tellinghuisen et al., 2005). So far only DI of NS5A has been crystalized and three crystal structures have been reported. These structures were obtained using different expression/ purification and crystallization conditions; however, all three showed formation of dimers through direct contact but in different conformations (Lambert et al., 2014; Love et al., 2009; Tellinghuisen et al., 2005). These differences suggest NS5A can interact with itself in multiple ways to form dimers or oligomers of different conformations and thus accommodate numerous functions and interactions with viral and/ or cellular proteins, ER membrane and nucleic acids (RNA) (Shanmugam et al., 2018). In a study using recombinant NS5A proteins expressed and purified from *E.coli* , four cysteines, C39, C57, C59 and C80 in the DI were found to be involved in NS5A dimerization through disulfide bridges and also were vital for viral replication and NS5A binding to RNA (Lim et al., 2012).

Domain II (DII) and domain III (DIII) are intrinsically unfolded and are relatively less conserved (Hanoulle et al., 2009; Liang et al., 2007). DII plays a role in replication through its interaction with cyclophilin A (CypA), a cellular protein that stimulates NS5A binding to RNA and regulates replication (Foster et al., 2011). DIII is dispensable for viral replication and large heterologous insertions and deletions can be done without affecting viral replication (Tellinghuisen et al., 2008). However, DIII is essential for virion assembly presumably through its direct interaction with core protein and localization to the assembly site (Appel et al., 2008).

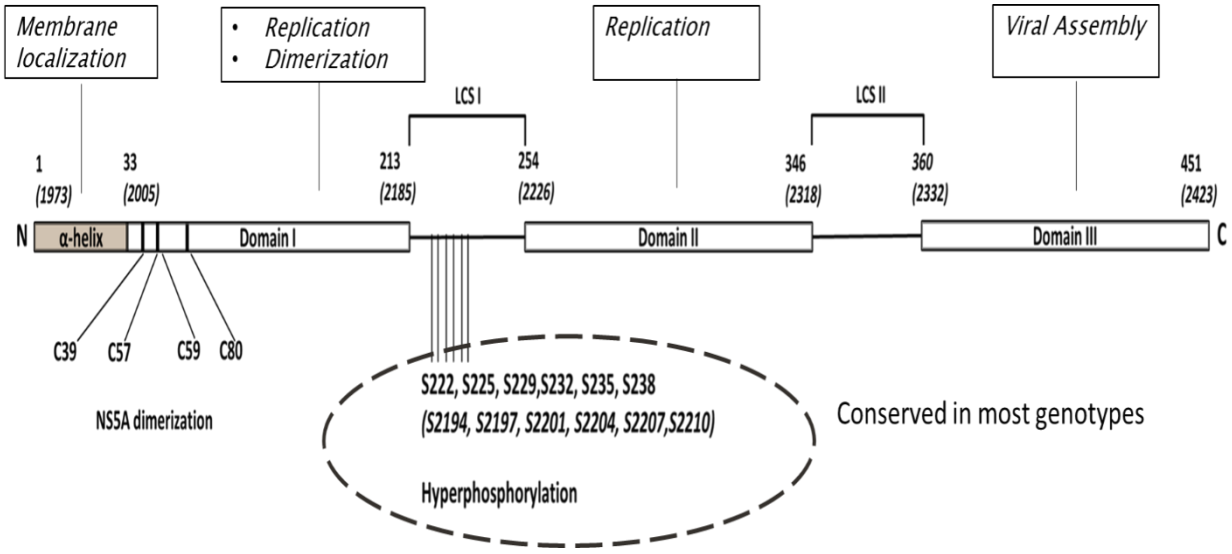

Figure 1.1.9: **Schematic design of HCV NS5A protein.** NS5A consist of N-terminal α-helix, three domains (domain I, II and III) linked by two low complexity sequences (LCS I and LCS II). Different regions of NS5A contribute to different viral functions. See text for details.

1.1.9.1 NS5A Phosphorylation

NS5A is a phospho-protein and exists in hypo- and hyper-phosphorylated forms displaying apparent molecular weight of 56 kDa (p56) and 58 kDa (p58) on SDS-PAGE. Hypo-phosphorylation of NS5A is mediated by several cellular protein kinases at the LCSII and the C terminus of DIII (Huang et al., 2007b). Hyperphosphorylation of NS5A is a highly regulated process which requires proper NS3-NS5A polyprotein processing and cleavage of NS5A protein in the same polypeptide (Koch and Bartenschlager, 1999; Neddermann et al., 1999b). Mass spectrometry, reverse genetics and phospho-proteomics studies have identified a highly conserved serine rich cluster in LCSI responsible for hyperphosphorylation. The phosphorylation on serine residues: S222, S225, S229, S232, S235 and S238 has been found to be the part of hyperphosphorylated NS5A (Fig. 1.1.9) (Hsu et al., 2018).

NS5A is known to literally interact with hundreds of host proteins (Macdonald and Harris, 2004). The plethora of protein-protein interaction by NS5A has been suspected to be mediated through its different phosphorylation states. For example, interaction of NS5A with

cellular proteins in particular the nucleosome assembly protein 1-like (NAP1L1), bridging integrator 1 (Bin1, also known as Amphiphysin II) and vesicle-associated membrane protein-associated protein A (VAP-A) is mediated through the phosphorylation of S225 as phosphorylation ablation mutation at S225 (S225A) disrupted this interaction and impaired viral replication and the subcellular localization of replication complexes (Goonawardane et al., 2017). NS5A can also interact with lipid kinase phosphatidylinositol 4-kinase type III alpha (PI4KIIIα) and activate it to stimulate its kinase activity i.e. conversion of phosphatidylinositol to phosphatidylinositol 4-phosphate (PI4P) and regulate the biogenesis of membranous web to allow viral replication (Reiss et al., 2011). In turn, active PI4KIIIα also regulates phosphorylation status of NS5A by favoring the synthesis of p56 or repressing the synthesis of p58 (Reiss et al., 2013). There are still multiple interactions of NS5A with host and viral (NS3-NS4B) proteins whose significance or role in regulation of NS5A hyperphosphorylation is not known.

Several serine/threonine kinases have been identified that are responsible for NS5A phosphorylation (Huang et al., 2007b; Macdonald and Harris, 2004). However, a link between specific phosphorylation sites with particular kinases and their role has not been established yet. One of the highly studied kinases is Casein kinase Iα (CKIα) because the spacing of the serine residues matches its phosphorylation preference i.e. three position distance between serine or threonine residues [(pS/ pT) XXS] (Flotow et al., 1990). Additional kinases involved in hyperphoshorylation of NS5A are polo-like kinases (Chen et al., 2010) and calmodulin-dependent kinases II (CAMK2) γ and δ (Chong et al., 2016).

NS5A hyperphosphorylation has been shown to play a role in virus replication and assembly (Appel et al., 2005; Chong et al., 2016; Masaki et al., 2014). In general, hyperphosphorylation of NS5A has been assumed to have a negative impact on HCV replication based on significant reduction of NS5A p58 synthesis in HCV with adaptive mutations (e.g., S2204I) (Blight et al., 2000). In addition, inhibition of p58 by kinase inhibitors has demonstrated an increase in HCV replication in the cell culture systems which further demonstrates the inverse relationship between NS5A hyperphosphorylation with HCV replication (Neddermann et al., 2004). Attempts have been made to understand the regulation and significance of NS5A hyperphosphorylation by studying the phosphorylation of individual serine resides in serine rich

cluster in LCS I. Through a mutational approach, it has been reported that phosphorylation ablating mutations at S225, S229, S232 and S235 depleted the replication of HCV-2a (JFH-1) (Chong et al., 2016; Goonawardane et al., 2017; Ross-Thriepland and Harris, 2014) while the same mutations enhanced the replication of HCV-1b (Con1) (Appel et al., 2005). This suggests that the role of NS5A hyperphosphorylation on replication may vary within the genotypes.

The serine residues in the hyperphosphorylation cluster are mostly three positions apart [(pS/ pT) XXS] and are suitable candidates for phosphorylation by CKIα (Flotow et al., 1990). Therefore,many researchers hypothesized that phosphorylation at these serine residues would occur in a sequential manner. In a recent phospho-proteomic study, evidence of sequential phosphorylation was shown with the help of pS232, pS235 and p238 antibodies (Hsu et al., 2018). In this study they showed that the phosphorylation at S232 mediates phosphorylation at S235 as a phospho-ablative mutation at S232 i.e. S232A in NS3-NS5B (HCV-2a, J6/ JFH-1) construct eliminated p235 species in hyper-phosphorylated NS5A. Similarly, the S235A eliminated the pS238 species in the hyper-phosphorylated NS5A. In both cases, when phosphorylation was ablated at S235 (S235A) and S238 (S238A), no effect of the phosphorylation at S232 and S235 was observed. Altogether, this suggests that sequential phosphorylation occurs from S232 to S235 and to S238. They also suggested that the priming phosphorylation at the S232 is mediated by CKIα as its knockdown and/or inhibition led to a significant decrease in the pS232 species in hyperphosphorylated NS5A (Hsu et al., 2018). However, it is important to note that in this study, pS238 species (albeit lower than in wild-type) was still detected in the S232A NS5A mutant, suggesting that either the phosphorylation of serine residues can also occur independent of sequential phosphorylation and/or there could be one or more phosphorylation priming site for sequential phosphorylation, for instance S229. This study gives insight into the regulation of NS5A hyperphosphorylation and at the same time highlights the complexity regarding regulation and significance of NS5A hyperphosphorylation.

NS5A is a multifunctional protein and phosphorylation of NS5A has been hypothesized to be behind it. Since the hyperphosphorylation sites in the NS5A are highly conserved and also play an important role in viral life cycle, these sites are attractive targets for the development of pan-genotypic DAAs.

1.1.10 HCV translation and NS5A

After entry in the hepatocytes, the HCV virion releases its positive sense, single-stranded RNA genome into cytoplasm which gets immediately translated to produce viral proteins to assist other stages of viral life cycle. The translation is initiated by the internal ribosomal entry site (IRES) located in the RNA 5'UTR (Tsukiyama-Kohara et al., 1992; Wang et al., 1993). The IRES facilitates the cap-independent translation of the viral RNA by the direct recruitment of HCV translation machinery factors: 40S ribosomal subunit, eukaryotic initiation factor (eIF2)/guanosine triphosphate/Met-transfer RNA complex and eIF3 to initiate translation (Pestova et al., 1998).

The HCV IRES also recruits several noncanonical proteins that supports optimal IRES activity, like the La protein, NSAP1, hnRNP-L and -D, IMP-1, Gemin5, LSm1-7 and PCBP2. Among them, La, NSAP1 and hnRNP-L, have sufficient evidence for their involvement in HCV RNA translation modulation while others have convincing to weak evidences (Niepmann, 2013). Among these IRES trans-acting factors (ITAFs), La protein has been shown to have high influence on HCV IRES activity as its activity has been found to be correlated with the expression of La protein i.e. knockdown of cellular La protein decreased while the expression enhanced the HCV IRES translation (Domitrovich et al., 2005; Shirasaki et al., 2010). Interestingly, La protein can interact with the HCV genome at both the 5' and 3'UTRs near the initiator AUG and within the poly(U/UC) region, respectively (Ali and Siddiqui, 1997; Spangberg et al., 1999). In addition, purified NS5A protein has been shown to interact with La protein (Houshmand and Bergqvist, 2003); however, the role of NS5A-La interaction in the infected cell and on the HCV life cycle has not been established yet.

HCV IRES-mediated translation has been suggested to be relatively inefficient as compared to that of other viruses with the IRES acting as a self-modulating mechanism to maintain a low level of replication and translation to promote viral persistence (Borman et al., 1995). In this regard, the viral core protein has also shown to inhibit the HCV translation by possibly binding to the stem-loop IIId domain, particularly a GGG triplet within the hairpin loop structure of the domain, within the IRES (Shimoike et al., 2006). Similarly, the essential viral protein NS5A has also shown to mediate viral translation; however, the mechanism and the

biological significance of the effect is still unclear due to numerous contradictory results being published (Gonzalez et al., 2009; He et al., 2003; Kalliampakou et al., 2005; Lourenco et al., 2008).

In our previous works, we further explored the role of HCV-1b NS5A in translation modulation and have found that NS5A downregulates the translation by binding to the poly(U/UC) region in the RNA 3'UTR (Hoffman et al., 2015a). All three domains except AH of NS5A were capable of downregulating the translation individually although to lesser extent than wild-type NS5A (Hoffman et al., 2015a). Predominantly, the residues R112, K312 and E446 of domain I, II and III were found to be involved in translation modulation by NS5A (Hoffman et al., 2015b, c). However, the effect of these residues on replication was different. For instance, alanine mutations at R112 impaired binding of domain I to poly(U/UC) region and was lethal for replication (Hoffman et al., 2015b), while the mutation E446A and double mutant K312A E446A significantly enhanced the replication (Hoffman et al., 2015c). In addition, residue R112 is conserved in all genotypes based on the Los Alamos HCV database, while K312 and E446 are not well conserved among HCV genotypes. Furthermore, the RNA binding affinity is different for all three domains with domain I and II being higher than domain III (Foster et al., 2010). All the divergences raise the question of whether the binding of NS5A to the poly(U/UC) region is the only mechanism behind translation downregulation by NS5A and also whether the role of NS5A on translation is conserved in all HCV genotypes.

HCV genome translation is an essential step for the viral existence and perseverance in the host. Understanding the mechanism and its regulation by host and viral proteins is important from a therapeutic aspect as blocking this process could also block viral replication and production of infectious viral particles.

2.0 HYPOTHESIS AND OBJECTIVES

2.1 Rationale I

The HCV NS5A protein is a multifunctional protein and found to play important role in viral replication and virus production. Extensive studies have been done to understand how NS5A regulates viral replication and virus production; however, its role in translation has not been well understood yet. As an attempt to understand the role of NS5A in HCV RNA translation, we previously studied the HCV-1b virus and its NS5A. We found that the HCV-1b NS5A downregulates viral RNA translation through a mechanism that requires NS5A to bind to the poly(U/UC) region in the 3' UTR. Also, all the three domains of the HCV-1b NS5A were capable of downregulating the translation, independently.

At present there are seven major genotypes of HCV and these have approximately 30% nucleotide difference throughout its genome including the region that codes for NS5A protein. This raises the question whether NS5A of different genotypes may affect its function. This question is of interest as previously studies have shown that NS5A may regulate functions differently depending on the genotype. For instance, NS5A is critical for replication of HCV of genotype 1b and 2a, however the mutation that decreases hyperphosphoylatin of the NS5A enhances replication in genotype 1b while the same mutations are detrimental for genotype 2a replication.

2.1.1 *Hypotheses*

i. HCV-2a NS5A downregulates the viral translation however, the mechanism could be different from HCV-1b NS5A

2.1.2 *Objectives*

i. Examine the effect of HCV-2a NS5A on translation of a HCV-2a reporter and genomic RNA

ii. Examine the significance of the IRES at the 5'UTR and the poly(U/UC) at 3' UTR on translation modulation by HCV-2a NS5A

iii. Determine the HCV-2a domains and respective regions involved in translation modulation

iv. Determine the mechanism behind translation modulation by HCV-2a NS5A

2.2 Rationale II

NS5A is a phospho-protein with two distinct phospho-species: hypo- and hyper-phosphorylated NS5A. The different phosphorylation states of NS5A have been thought to mediate its interaction with multiple host proteins and thus regulate different stages of viral life cycle. Based on mass spectrometry, reverse genetics and phospho-proteomics studies a highly conserved serine rich cluster in the low complexity sequence I (LCSI) of NS5A is responsible for NS5A hyperphosphorylation. The serine residues involved in NS5A hyperphosphorylation are S222, S225, S229, S232, S235 and S238. The significance of NS5A hyperphosphorylation has been studied in terms of replication and viral assembly and has been assumed to have a negative impact on them. However, no attempts have been done to study the role of NS5A different phosphorylation in viral RNA translation.

2.2.1 *Hypotheses*

i. Our previous work has found that NS5A can downregulate the HCV-1b RNA translation. There is the possibility that the NS5A modulates the translation through its different phosphorylation states. Therefore, we hypothesized that phosphorylation on at least one or more of the serine residues involved in NS5A hyperphosphosphorylation modulates the viral RNA translation.

2.2.2 *Objectives*

i. Determine the effect of hypo- and hyper-phosphorylated NS5A on HCV-1b genomic RNA translation

ii. Determine the role of phosphorylation at each individual serine residues involved in NS5A hyperphosphorylation i.e. S225, S229, S232, S235 and S238

iii. Determine whether hyperphosphorylation of NS5A affects its dimerization

3.0 HCV-2a NS5A downregulates viral translation predominantly through domain I

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Keywords: HCV-2a NS5A; HCV RNA translation; NS5A domain I; La protein

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3.1 Authors' contribution

All the experiments within this chapter were performed by Mangyung Kandangwa. The manuscript was written by Mangyung Kandangwa and edited by Qiang Liu.

3.2 Abstract

Hepatitis C virus (HCV) non-structural protein NS5A is a multifunctional protein with critical roles in viral replication and assembly. Previously, our group showed that HCV-1b NS5A downregulates viral translation through binding to the poly(U/UC) tract in the 3'UTR. As NS5A of different HCV genotypes may have different functions or carry out the same function through genotype-specific mechanisms, we investigated the effect of HCV-2a NS5A on viral translation. We found that although HCV-2a NS5A also downregulates viral translation, it does not require the poly(U/UC) region in 3'UTR. This is different from HCV-1b NS5A. The translation downregulation by HCV-2a NS5A was predominantly mediated by its domain I. Moreover, we showed that knocking down La, an IRES trans-acting factor, significantly enhanced translation downregulation by NS5A. Our results elucidated how HCV-2a NS5A modulates viral translation.

3.3 Introduction

Hepatitis C virus (HCV) infection affects 71 million people worldwide (WHO Hepatitis C Fact Sheet, July 2019. https://www.who.int/news-room/fact-sheets/detail/hepatitis-c). HCV infection often leads to chronic hepatitis and can progress to liver cirrhosis and hepatocellular carcinoma (Di Bisceglie, 1997; Giannini and Brechot, 2003). HCV belongs to the genus hepacivirus of the *Flaviviridae* family (Dustin and Rice, 2007). Seven major HCV genotypes (1-7) are classified on the basis of phylogenetic and sequence analyses of the whole viral genome (Smith et al., 2014a). Viruses of different genotypes often exhibit different replication capacities and pathogenesis. The HCV genome is positive-sense single-stranded RNA of 9.6 kb and encodes a polyprotein precursor of approximately 3000 amino acids. On the ribosomes in the rough endoplasmic reticulum (ER), the HCV polyprotein is synthesized and co- and post-translationally cleaved by viral and host proteases to yield three structural proteins (core, E1 and E2) and six non-structural proteins (NS2 to NS5B) (Bartenschlager and Lohmann, 2000; Dustin and Rice, 2007). The ends of the viral genome contain highly structured untranslated regions (UTRs). These UTRs are involved in translation and replication modulation.

The 5'UTR consist of internal ribosome entry site (IRES) which initiates translation of HCV RNA (Tsukiyama-Kohara et al., 1992; Wang et al., 1993). The 3'UTR is a unique non-poly(A) tail and consists of a short genotype-specific variable region, a pyrimidine tract of variable length (poly(U/UC)), and a conserved 98-nucleotide sequence, known as the X-tail region (Kolykhalov et al., 1996). The X-tail region is composed of three stem-loops structures: SL1, SL2 and SL3. The variable region, poly(U/UC) tract, and SL1 of X-tail region have been found to contribute to translation enhancement but the mechanism is still unclear (Song et al., 2006). Some of the proposed mechanisms include long range RNA-RNA interaction with IRES, recruitment of viral and/or cellular factors which may mediate 5' and 3'UTR interaction and ribosome recycling between the two UTRs during successive rounds of translation (Bai et al., 2013; Hoffman and Liu, 2011).

Among the HCV viral proteins, non-structural protein 5A (NS5A) is a proline-rich phosphoprotein consisting of an N-terminal amphipathic α-helix (AH) and three domains (Ross-Thriepland and Harris, 2015). The three domains are connected by two inter-domain low

complexity sequences (LCSs), LCS I and LCS II. NS5A has been demonstrated to have multiple functions. It is essential for replication and virus particle assembly. It is also involved in several cellular processes, such as interferon resistance and apoptotic regulation (McGivern and Lemon, 2011). Mechanistically, NS5A has been shown to function through its interaction with other HCV proteins and genomic RNA, as well as host factors (Huang et al., 2007a). Recent research of our group and others has studied the role of NS5A in RNA translation modulation. These studies showed that NS5A downregulates viral translation of HCV-1a and HCV-1b (Hoffman et al., 2015a; Kalliampakou et al., 2005). We further showed that all three individual domains are capable of downregulating viral RNA translation (Hoffman et al., 2015b, c). Moreover, we showed that the binding of NS5A to the poly(U/UC) region in the HCV 3'UTR is required for the downregulation (Hoffman et al., 2015a).

HCV IRES activity is regulated by IRES trans-acting factors (ITAFs) (Niepmann, 2013). For example, La protein has been shown to be able to regulate HCV RNA translation as HCV IRES activity has been found to be correlated with the La protein level (Domitrovich et al., 2005; Shirasaki et al., 2010). Knockdown of cellular La protein decreases, while the ectopic expression enhances HCV translation. How La protein regulates HCV translation is not clear. Interestingly, La protein can interact with the HCV genome at both 5' and 3'UTRs near the initiator AUG and within the poly(U/UC) region, respectively (Ali and Siddiqui, 1997; Spangberg et al., 1999). In addition, NS5A protein has been shown to interact with La protein *in vitro* (Houshmand and Bergqvist, 2003). These observations raise a possibility that NS5A-La interaction may play a role in modulating viral translation.

NS5A possesses functional differences in replication and virus production based on its genotype (Scheel et al., 2012). As an effort to understand the genotype specific NS5A role in HCV translation, we characterized the regulatory role of HCV-2a NS5A in translation in this study. We found that HCV-2a NS5A downregulates viral translation via a mechanism that does not require the poly(U/UC) region in 3' UTR. Mapping experiments demonstrated that domain I is the major domain able to inhibit translation. These results suggest a different mechanism from the HCV-1b NS5A. Furthermore, we showed that the inhibitory effect of NS5A on HCV

translation is significantly enhanced by knocking down La protein, suggesting that interaction of NS5A-La may be involved in HCV translation downregulation by NS5A.

3.4 Results

3.4.1 HCV-2a NS5A downregulates viral RNA translation

We previously showed that HCV-1b NS5A downregulates HCV-1b RNA translation by binding to the poly(U/UC) region of 3'UTR (Hoffman et al., 2015a). To determine whether the role of NS5A is conserved in genotype 2a, we co-transfected Huh-7 cells with HCV-2a NS5A or control protein EGFP expression plasmids and monocistronic HCV-2a translation luciferase reporter RNAs with either wild-type (WT) or poly(U/UC) deletion (Δpoly(U/UC)) 3'UTR. As shown in Fig. 3.1a, HCV-2a NS5A significantly downregulated the translation of viral RNA with WT 3'UTR. Somewhat to our surprise, NS5A also downregulated the translation of HCV-2a RNA Δpoly(U/UC) 3'UTR (Fig. 3.1b). To substantiate the results obtained with the monocistronic RNA translation reporters, we used HCV-2a replication-deficient genomic RNAs with the rLuc gene in between p7 and NS2 with WT or Δpoly(U/UC) 3'UTR (Fig. 3.1c and d). NS5A expression led to a decrease in translation of both types of RNAs (Fig. 3.1c and d). The results demonstrated that, unlike HCV-1b NS5A, HCV-2a NS5A downregulates viral RNA translation irrespective of the poly(U/UC) sequence in 3'UTR. NS5A and EGFP protein expression was demonstrated in Western blotting (Fig. 3.1g).

Next, we asked the question as to whether the inhibitory effect of NS5A on translation and the requirement for the poly(U/UC) sequence are conferred by the NS5A protein or the UTR sequences of HCV-1b and -2a. To answer this question, we co-transfected HCV-1b translation rLuc reporter RNAs with either WT or Δpoly(U/UC) 3'UTR, together with HCV-2a NS5A or EGFP expression plasmids. We found that HCV-2a NS5A was able to inhibit the translation of both RNAs in comparison to control EGFP protein (Fig. 3.1e and f). These results indicated that HCV-2a NS5A inhibits HCV RNA translation through a mechanism that does not require the presence of the poly(U/UC) sequence.

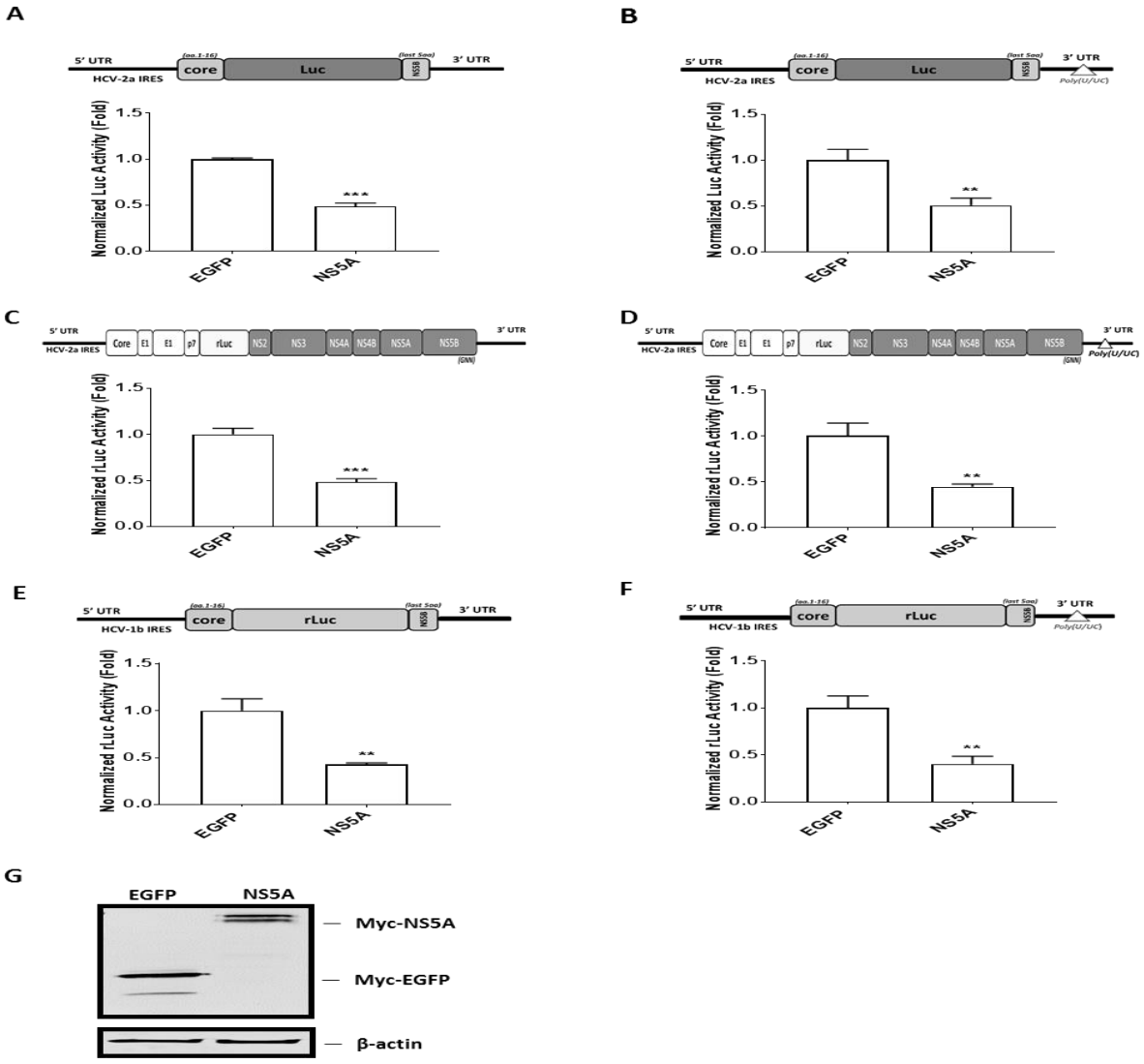

Figure 3. 1: **HCV-2a NS5A downregulates viral RNA translation.** (A, B) Huh-7 cells were co-transfected with a monocistronic translation reporter RNA with WT 3'UTR (A) or with poly(U/UC) deleted (Δpoly(U/UC)) 3'UTR (B) with the plasmids expressing Myc-tagged EGFP or HCV-2a NS5A. Cells were harvested 24 hr post-transfection and luciferase assay performed. (C, D) Huh-7 cells were co-transfected with HCV-2a genomic GNN RNA with WT 3'UTR (C) or Δpoly(U/UC) 3'UTR (D) with plasmids expressing Myc-tagged EGFP or HCV-2a NS5A. Cells were harvested after 8 hr post-transfection for luciferase assay. (E, F) Huh-7 cells were co-transfected with the HCV-1b monocistronic translation reporter RNA with WT 3'UTR (E) or Δpoly(U/UC) 3'UTR (F) with plasmids expressing Myc-tagged EGFP and HCV-2a NS5A. At 24 hr post-transfection, cells were harvested and luciferase assay performed. The luciferase value of RNA with EGFP expression was set to 1. ** if $P < 0.01$, *** if $P < 0.001$. (G) Expression of EGFP and HCV-2a NS5A demonstrated by Western blotting using a Myc-tag antibody. The levels of β-actin were also determined using a β-actin-specific antibody.

3.4.2 Domain I of NS5A downregulates HCV translation similar to WT NS5A

NS5A consists of an N-terminal amphipathic α-helix (AH) and domains I, II and III with two inter-domain LCSs. To map the regions of NS5A responsible for its translation modulatory effect, plasmids encoding NS5A with an AH deletion or the individual domains of NS5A (Fig. 3.2a) were utilized along with the monocistronic RNA reporters with WT or Δpoly(U/UC) 3'UTR. Expression of NS5A with AH deletion downregulated the translation of viral RNA with WT 3'UTR similar to the full-length WT NS5A (Fig. 3.2b). In the case of viral Δpoly(U/UC) 3'UTR RNA, AH deletion was still able to inhibit viral translation, but to a lesser degree than WT NS5A (Fig. 3.2c). Expression of domain I-LCS I did not downregulate the translation of either RNAs. In contrast, domain I itself was able to downregulate the translation of both RNAs to similar level as the WT NS5A (Fig. 3.2b and c). While domain II-LCS II could not downregulate the translation of both viral RNAs, domain II itself was found to modestly downregulate the translation of the RNA with WT 3'UTR, but had no effect on the translation of viral RNA with Δpoly(U/UC) 3'UTR. Lastly, domain III had no effect on the translation of both RNAs. The expression levels of NS5A and its domain constructs are demonstrated in Western blotting (Fig. 3.2d).

Taken together, these results indicated that domain I plays a dominant role in modulating viral RNA translation by HCV-2a NS5A.

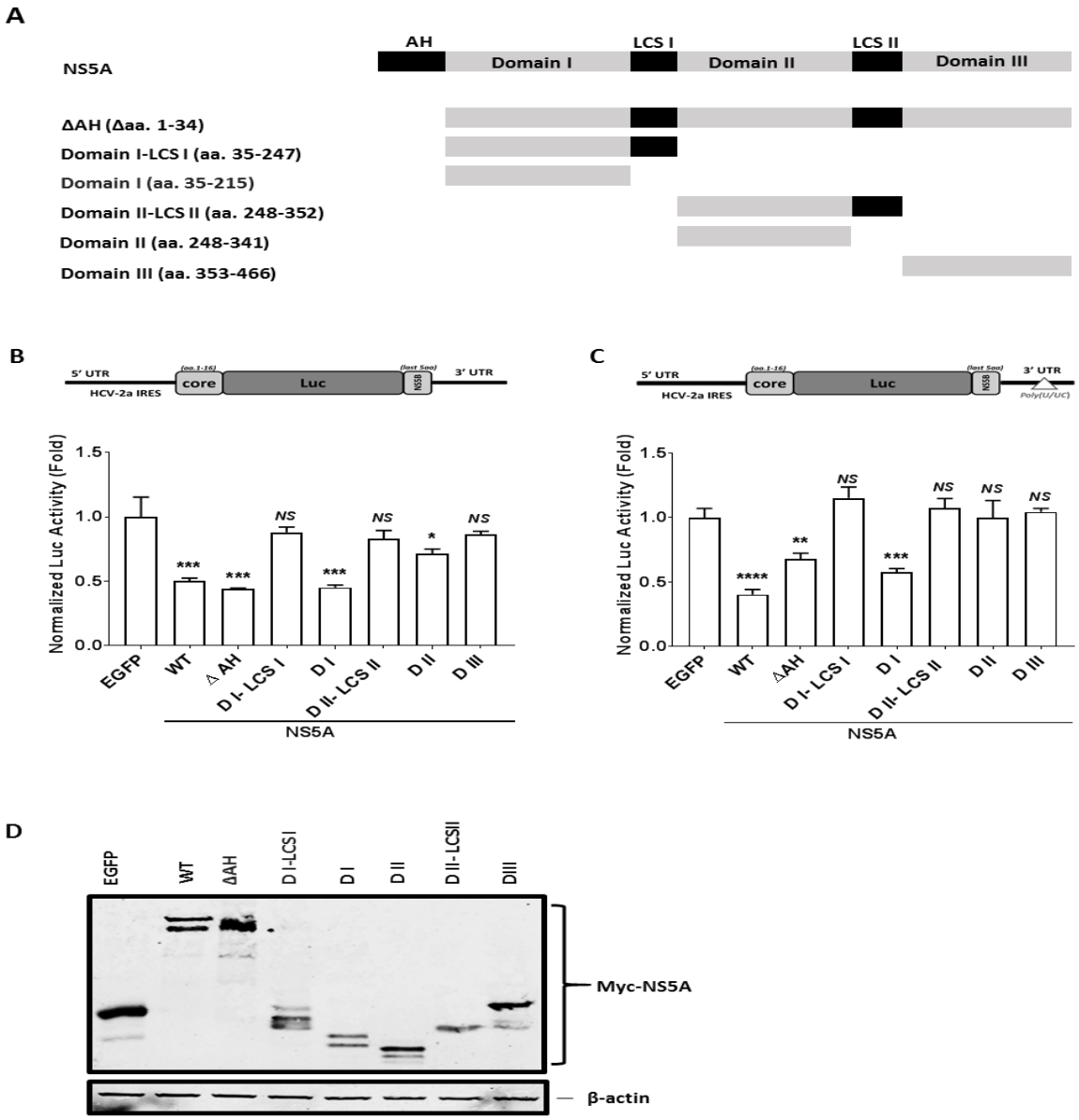

Figure 3. 2: **Domain I of HCV-2a NS5A downregulates viral translation.** (A) Schematic diagram of NS5A domains and NS5A mutants that were used. (B, C) Huh-7 cells were co-transfected with plasmids expressing Myc-tagged EGFP, full-length WT NS5A or truncated NS5A domains with either HCV-2a translation reporter RNA with WT 3'UTR (B) or with Δpoly(U/UC) 3'UTR (C). Cells were harvested 24 hr post-transfection and luciferase assay performed. The luciferase value obtained after EGFP expression was set to 1. * if $P < 0.05$, ** if $P < 0.01$, *** if $P < 0.001$, **** if $P < 0.0001$, *NS* if not significant. (D) Expression of Myc-tagged EGFP, full-length NS5A and NS5A mutant proteins was demonstrated by Western blotting using a Myc-tag antibody. The levels of β-actin were also determined using a β-actin-specific antibody.

3.4.3 Mapping NS5A domain I amino acid sequences that downregulate viral translation.

Results presented so far showed that domain I can downregulate HCV RNA translation with WT and Δpoly(U/UC) 3'UTR similar to the full-length NS5A. To determine the regions of domain I involved in viral translation modulation, we generated two domain I (DI) truncations by deleting 60 amino acids from either the N- or C-termini (aa. 95-215 and aa. 35-154, Fig. 3.3a) and used them in translation assay. As shown in Fig. 3.3b, neither truncated DI proteins could downregulate viral translation, suggesting that the deleted 60 amino acids are involved in modulating viral translation. To confirm this, we generated constructs expressing these two 60 amino acid-long fragments (aa. 35-94, and 155-215, Fig. 3.3a). We also generated a construct expressing the middle fragment spanning aa. 95-154 (Fig. 3.3a). As shown in Fig. 3.3d, all these three individual fragments were able to downregulate viral translation. These results suggested that domain I can be divided into three 60 amino acid regions that can either individually or all three together downregulate viral translation. The expression of these domain I truncations are demonstrated by Western blotting (Fig. 3.3c and e).

3.4.4 S146 is involved in viral translation modulation by NS5A domain I.

HCV-2a NS5A has a serine residue at 146 which is absent in the majority of genotypes including HCV-1b. It has been shown previously that phosphorylation of the serine residue at position 146 in domain I regulates NS5A hyperphosphorylation (Ross-Thriepland and Harris, 2014). To determine whether S146 phosphorylation is involved in translation modulation by domain I, we generated phosphoablatant (S146A) and phosphomimetic (S146D) mutations at this position in the context of domain I (Fig. 3.4a) and measured the translation of viral RNAs with WT and Δpoly(U/UC) 3'UTR. We found that both the NS5A domain I S146A and S146D mutants were capable of significantly downregulating the translation of both viral RNAs in comparison to control (Fig. 3.4b and c). However, when compared to the WT domain I, the two S146 mutants resulted in significantly lesser, but rather modest, inhibition on translation (Fig. 3.4b and c). These results suggested that, rather than the changes in the charge conferred by S146A and S146D mutations, the integrity of S146 may be important for the domain I of NS5A

to downregulate viral RNA translation. The expression of S146A/D domain I mutant proteins is shown in Western blot (Fig. 3.4d).

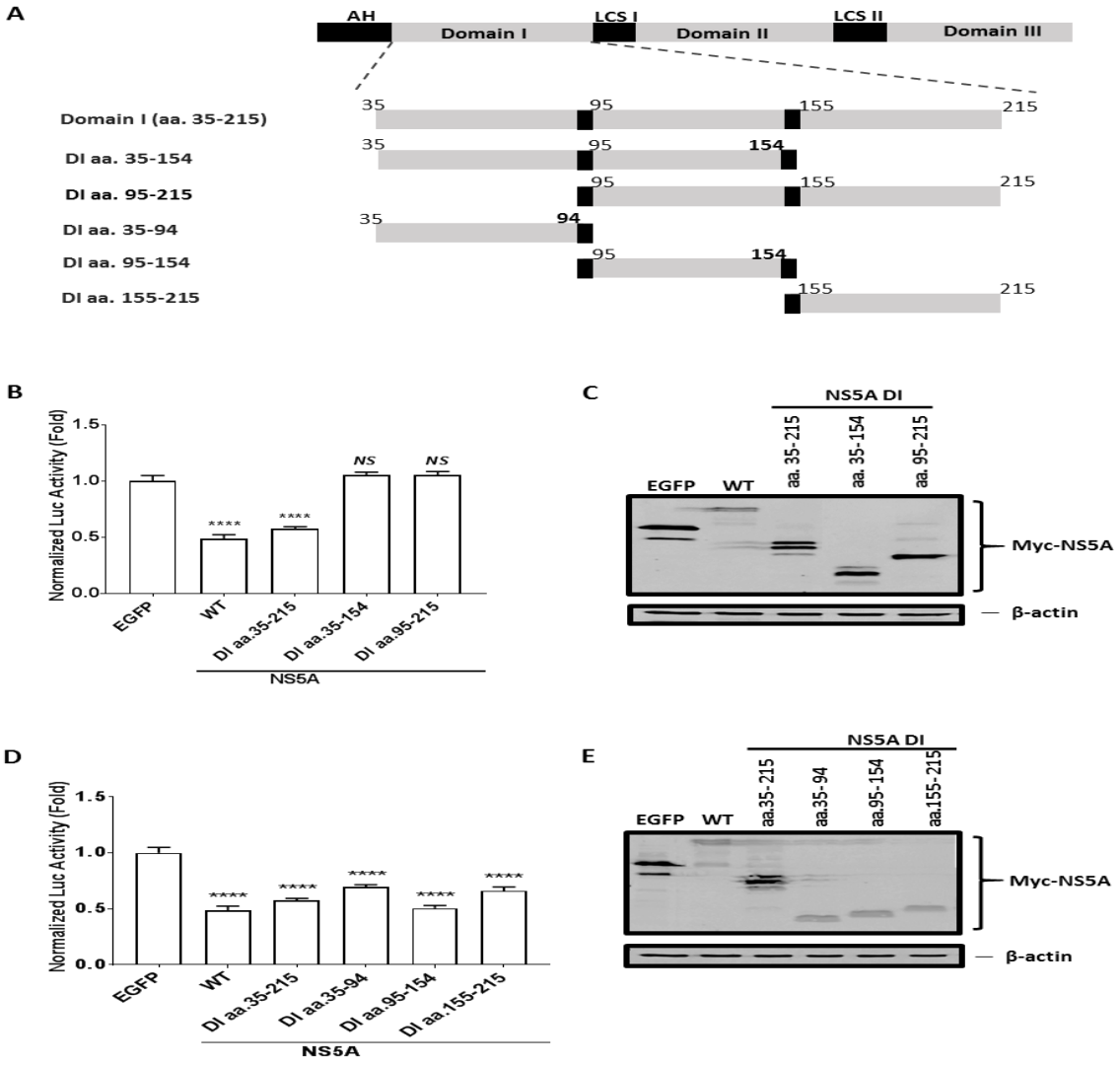

Figure 3. 3: **Searching for regions in domain I that can modulate viral RNA translation.** (A) Schematic diagram showing NS5A domain I and its truncated mutants that were used. (B, C) In Huh-7 cells, HCV-2a translation Luc reporter RNA with WT 3'UTR was co-transfected with plasmids expressing either Myc-tagged EGFP, full-length WT NS5A, domain I or two 60-aa. deletional mutants (B) or plasmids expressing three 60-aa. fragments (D). Luciferase assay was performed 24 hr post-transfection. **** if *P* <0.0001, *NS* if not significant. (C and E). Expression of Myc-tagged EGFP, NS5A domain I and mutant proteins was demonstrated by Western blotting using a Myc-tag antibody. The levels of β-actin were also determined using a β-actin-specific antibody.

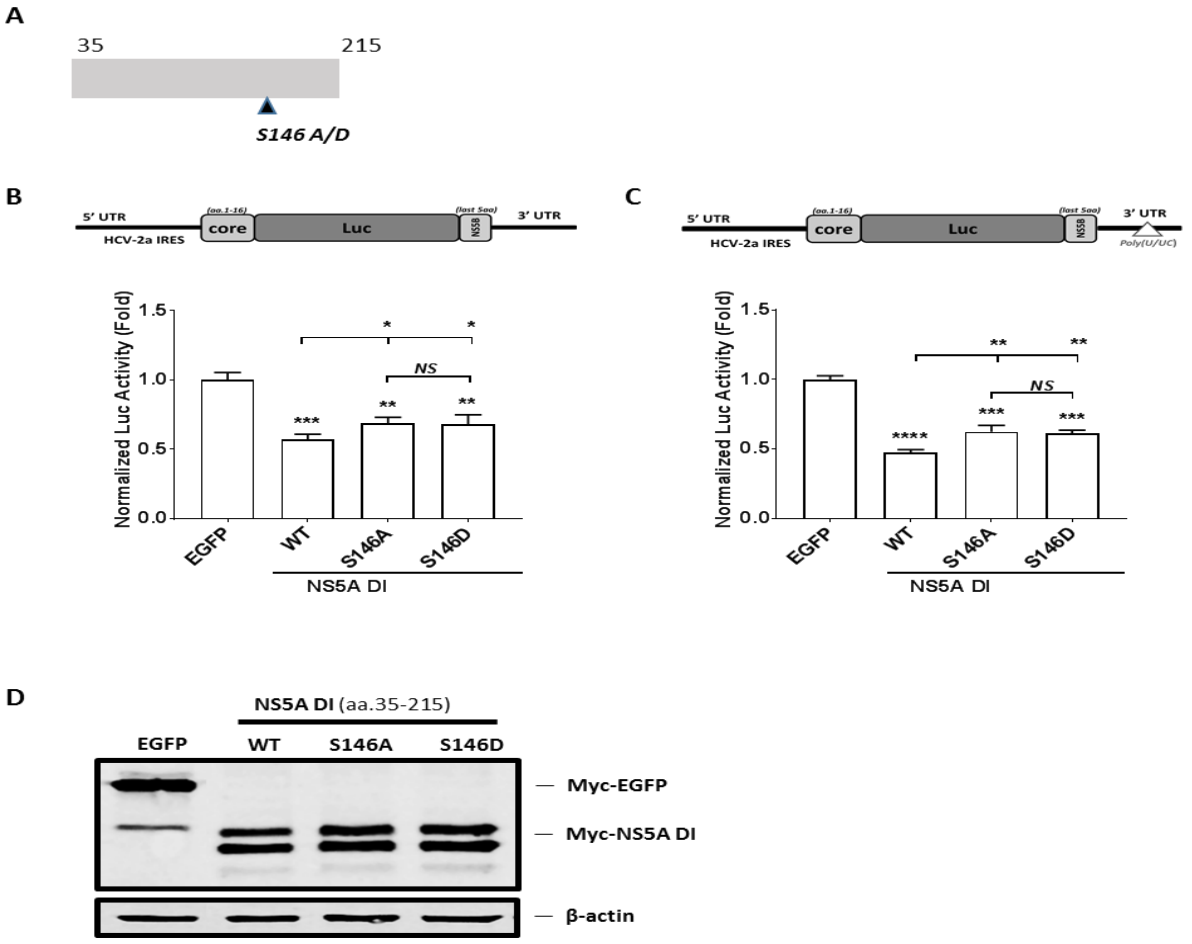

Figure 3. 4: **S146 in domain I is involved in viral translation modulation.** (A) Schematic diagram of domain I and the location of S146 mutations. In Huh-7 cells, plasmids expressing either Myc-tagged EGFP, wild-type domain I, S146A, or S146D, were co-transfected with HCV-2a translation Luc reporter RNAs with WT 3'UTR (B) or Δpoly(U/UC) 3'UTR (C). Luciferase assay was performed 24 hr post-transfection. The luciferase value obtained after EGFP expression was set to 1. * if $P < 0.05$, ** if $P < 0.01$, *** if $P < 0.001$, **** if $P < 0.0001$, *NS* if not significant. (D) Expression of Myc-tagged EGFP, domain I, domain I S146A and S146D proteins was shown by Western blotting with a Myc-tag antibody. The levels of β-actin were also determined using a β-actin-specific antibody.

3.4.5 La protein is involved in viral translation downregulation by NS5A

Both NS5A and La proteins bind to HCV UTRs, and La protein positively regulates viral translation (Ali and Siddiqui, 1997; Huang et al., 2005; Spangberg et al., 1999). In addition, it has been shown that NS5A and La proteins interact with each other (Houshmand and Bergqvist, 2003). As such, we hypothesized that NS5A may downregulate viral translation by interrupting the positive regulation by La protein. To test this hypothesis, we first used a La-specific shRNA to knock down La protein level and studied the effect on translation. A non-silencing shRNA was used as a control. Knocking down La shRNA resulted in significantly lower Luc level in comparison to control shRNA (Fig. 3.5a). This is consistent with the published positive effect of La on HCV translation (Shirasaki et al., 2010). Then cells were co-transfected with an NS5A protein expression plasmid and La shRNA. Plasmid expressing EGFP was included as a control. Results showed that, when La was knocked down, viral translation was further downregulated significantly after NS5A expression in comparison to EGFP control (Fig. 3.5a). These data indicated that knocking down La and over-expressing NS5A have a synergistic inhibitory effect on viral translation. The levels of La protein after shRNA knockdown as well as EGFP and NS5A were demonstrated by Western blotting (Fig. 3.5b).

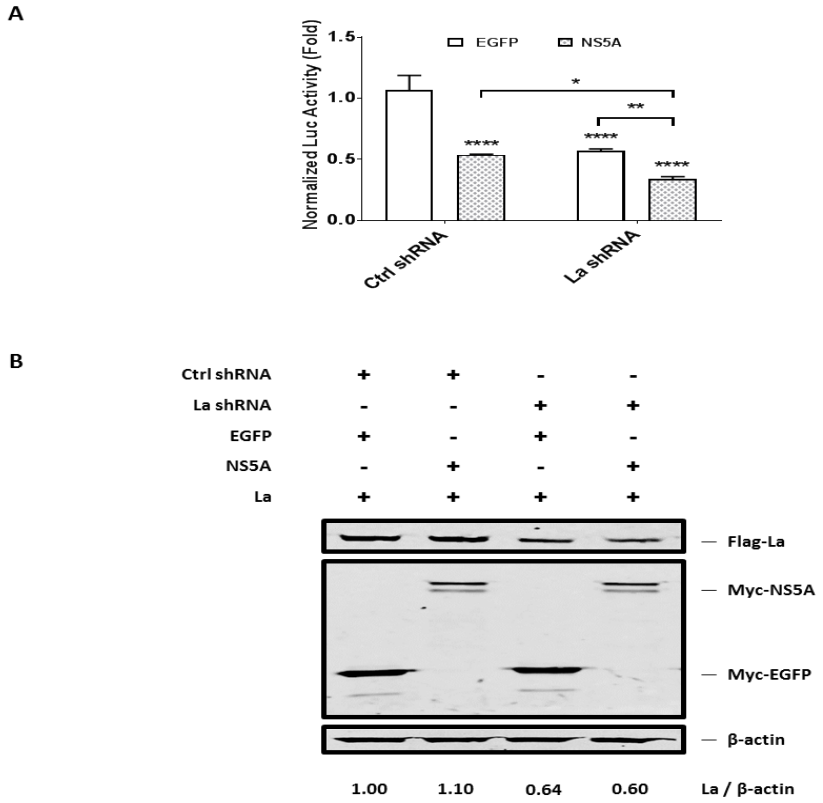

Figure 3. 5: **Knocking down La enhances translation downregulation by NS5A.** (A) Huh-7 cells were co-transfected with HCV-2a translation Luc reporter RNA, plasmids expressing Myc-tagged EGFP or NS5A, and plasmids encoding non-silencing shRNA or shRNA targeting La. Cells were harvested 24 hr post-transfection and luciferase assay performed. The luciferase value from non-silencing shRNA and EGFP expression was set to 1. * if $P < 0.05$, ** $P < 0.01$, *** if $P < 0.001$, **** if $P < 0.0001$, *NS* if not significant. (B) To demonstrate the knocking down of La by shRNA, Huh-7 cells were co-transfected with plasmids expressing Myc-tagged EGFP or NS5A, plasmids expressing Flag-tagged La, and plasmids encoding non-silencing shRNA or shRNA targeting La. The levels of EGFP, NS5A, and La proteins were demonstrated in Western blotting with Myc-tag and Flag-tag antibodies, respectively. The levels of β-actin determined with a β-actin antibody were used in normalizing the La protein levels in densitometry analysis shown underneath the blot.

3.5 Discussion

HCV NS5A is a multifunctional protein that plays a key role in HCV life cycle. It is essential for viral replication and required for viral assembly. Recent research of our group focused on understanding the effect of NS5A on viral translation. We showed that HCV-1b NS5A downregulates the HCV-1b RNA translation by binding to the poly(U/UC) tract in 3'UTR (Hoffman et al., 2015a). All three individual domains can downregulate viral translation (Hoffman et al., 2015b, c). In this study, we found that HCV-2a NS5A also downregulates RNA translation of both HCV-1b and -2a, suggesting a well conserved function of NS5A. However, different from HCV-1b NS5A, translation downregulation by HCV-2a NS5A does not require the presence of the poly(U/UC) sequence in 3'UTR. Domain mapping experiments showed that domain I is equally effective as the full-length NS5A in inhibiting viral translation irrespective of the poly(U/UC) sequence. Domain III has no effect. Interestingly, although far less effective in downregulating viral translation, domain II of HCV-2a NS5A appears to exert this function only when the poly(U/UC) sequence is present, reminiscent of how HCV-1b NS5A regulates translation. These results suggest both unique and overlapping functions of NS5A - poly(U/UC) RNA interaction of different genotypes. It is also important to note that NS5A proteins of HCV-1b and HCV-2a have different affinity to 3'UTR RNA with HCV-2a NS5A being ~50% lower than the HCV-1b NS5A (Foster et al., 2010). All three domains of both genotypes of NS5A are capable of binding to the 3'UTR, but again the affinity of each domains is different. For HCV-1b, RNA binding affinity of domains I and II is similar and significantly higher than that of domain III. In the case of HCV-2a NS5A, RNA binding affinity for domain II is the highest, domain III is the lowest, and domain I is intermediate. This difference in RNA-binding of NS5A of different genotypes may result in different mechanisms for a certain function of NS5A.

NS5A has been found to possess genotype- or isolate specific functional differences in viral replication and virus production (Scheel et al., 2012). In addition, the conserved site or residue in the NS5A could also have different role depending on its genotype (Scheel et al., 2012). This further explains the possible reason behind the difference in the translation modulation by HCV-2a NS5A from HCV-1b NS5A. Furthermore, there is approximately 40% protein sequence difference between NS5A of HCV-1b (the N strain) and HCV-2a (the JFH-1

strain) and HCV-2a NS5A has additional 15 aa. This difference could also contribute to discrepancy in their structural conformation and thus lead them to function differently. To support this hypothesis, comparison of NS5A crystals structure of these genotypes would be desirable. However, crystal structure for HCV-2a NS5A is not available at present.

HCV-2a NS5A domain I (aa. 95-215) modulated viral translation as effectively as the full length WT NS5A (Fig. 3.3b and d). All three 60 amino acids domain I truncated proteins: aa. 35-94, aa. 95-154 and aa. 155-215, could also downregulate viral translation (Fig. 3.3d). Surprisingly, addition of aa. 35-94 or aa. 155-215 to aa. 95-154 rendered the proteins i.e. DI aa. 35-154 and DI aa. 95-215, unable to downregulate viral translation (Fig. 3.3b). The reasons behind different translation modulation by the 60 aa and 120 aa domain I truncated proteins are not clear. One possibility is that these truncated domain I fragments have adopted different conformations. It is tempting to speculate that the structural conformation of 120 aa domain I truncations may not be ideal for its interaction with the host factors involved in translation downregulation while this was supported by the conformation of 60 aa domain I truncations.

NS5A exists in hypo- and hyper-phosphorylated forms (Huang et al., 2007a; Ross-Thriepland and Harris, 2015). The phosphorylation states of NS5A have been hypothesized to act as a molecular switch regulating the different functions of NS5A (Appel et al., 2005; Ross-Thriepland and Harris, 2014). Genetic mutation and phosphoproteomics studies have pinpointed a cluster of serine residues (S222, S225, S229, S232, S235, and S238) in the LCS I region responsible for N5SA hyperphosphorylation (Hsu et al., 2018; Ross-Thriepland and Harris, 2014). In our study, we found that HCV-2a NS5A domain I downregulated the viral translation. Interestingly, however, addition of LCS I to domain I resulted in a loss of this function. The underlying mechanism is not immediately clear, but it is possible that phosphorylation in the serine cluster in LCS I may have changed the protein conformation significantly, affecting domain I - host factor interactions involved in RNA translation downregulation. In addition to the serine cluster in LCS I, phosphorylation of S146 in domain I has been demonstrated (Ross-Thriepland and Harris, 2014). Phosphomimetic mutation (D) on the S146 residue causes a reduction in hyper-phosphorylation, while phosphoablant mutation (A) on S146 has no effect, suggesting that phosphorylated S146 is not a component of hyper-

phosphorylated NS5A, but negatively regulates hyperphosphorylation (Ross-Thriepland and Harris, 2014). Neither S146D nor S146A affects HCV replication (Ross-Thriepland and Harris, 2014). When we used these two S146 mutants in a translation assay, both mutants can still downregulate translation, but to a lesser degree than the wild-type domain I. These data suggest that the integrity of the S146, rather than the charge changes conferred by mutations mimicking its phosphorylation states, may have a role in translation modulation.

Although La protein has been shown to be required for HCV IRES-mediated translation, the biological significance of La-NS5A interaction has not been well studied (Ali et al., 2000; Shirasaki et al., 2010). Our results showed that knocking down La significantly enhances translation inhibition by NS5A, implicating La in translation modulation by NS5A. The molecular mechanisms are not clear at this moment. Since both La and NS5A bind to HCV UTR sequences, and the two proteins interact with each other, one plausible mechanism is that there is a competition in UTR-binding and thereby regulates translation. Further study is required to investigate the mechanism.

In conclusion, we have demonstrated that HCV-2a NS5A downregulates the viral RNA translation predominantly through its domain I irrespective of the presence of the poly(U/UC) sequence in viral 3'UTR. This is different from HCV-1b NS5A protein. We also showed that La protein is involved in translation modulation by HCV-2a NS5A. Our research advanced our understanding of the functions of HCV NS5A.

3.6 Materials and Methods

3.6.1 Plasmids, RNA reporters, and in vitro transcription

All plasmids were constructed as per standard methods and confirmed by DNA sequencing. To generate the plasmids expressing EGFP, NS5A, individual NS5A domains and different domain I truncations with a Myc-tag, the respective coding sequences were amplifed by PCR and cloned into the pEF vector. Similarly, a plasmid expressing human La protein with a Flag-tag and a lentiviral plasmid expressing La-specific shRNA with the target sequence 5' TGCTAAGAAATTTGTAGAGAC 3' were constructed (Domitrovich et al., 2005). The HCV-2a J6/JFH (p7-RLuc2A) GNN construct is a full length replication-deficient viral genome with rLuc2A insertion between p7 and NS2 (Jones et al., 2007). Another construct was made upon deleting the poly(U/UC) sequence in 3'UTR. The HCV-2a monocistronic RNA translation reporter construct contained the HCV-2a JFH-1 5'UTR, sequence encoding the first 16 aa of the core protein, an internal Luc gene, sequence encoding the last 5 aa of the NS5B and the 3'UTR. This reporter was used to develop another reporter with the poly(U/UC) tract deleted in 3'UTR. HCV-1b translation reporter constructs have been described previously (Hoffman et al., 2015a). Plasmids were linearized by *Kpn*2I (ThermoFischer Scientific) or *Xba*I (New England Biolabs), respectively and then they were *in vitro* transcribed using MEGAscript T7 transcription kit (Ambion).

3.6.2 Cell lines, transfections and luciferase assay

Huh-7 cells were maintained in Dulbecco's modified Eagle's medium (MilliporeSigma) supplemented with 10% (v/v) FBS (MilliporeSigma) and 1% penicillin-streptomycin and cultured at 37 °C and 5% CO_2. Cells were co-transfected with DNA and/or RNA using Jet-PEI transfection reagent (Polyplus-Transfection). For luciferase assay, cells were lysed with Passive Lysis Buffer and Luc/rLuc activities were determined using the GloMax 20/20 Luminometer according to the manufacturer's instructions (Promega). The luciferase readings were normalized to total protein concentration determined by Bradford protein assay (Bio-Rad).

3.6.3 Western blotting and antibodies

Cell lysates were subjected to 10% or 12% SDS-PAGE and transferred onto nitrocellulose membranes. The membranes were blocked with 5% skimmed milk in PBS for 1 hr at room temperature before incubation with a primary antibody overnight at 4 °C. After washing, the membranes were then incubated with an appropriate infrared dye-labelled secondary antibody for 1 hr at room temperature. The membranes were then washed with PBST (PBS + 0.1% Tween 20) and scanned using Odyssey CLx Imaging System (Li-Cor Biosciences). Anti-β-actin, anti-Myc antibodies were purchased from Cell Signaling Technology. Anti-Flag antibody was from Sigma-Aldrich. Secondary antibodies IRDye 800 CW goat anti-mouse IgG and IRDye 680 goat anti-rabbit IgG were from Li-Cor Biosciences.

3.6.4 Statistical analysis

All the experiments were done in triplicates and the experimental data were analysed using the GraphPad Prism 7. Statistical differences were determined by Student's *t*-test or two-way ANOVA. Statistical significance was demonstrated as follows: * if $P < 0.05$, ** if $P < 0.01$, *** if $P < 0.001$, **** if $P < 0.0001$, *NS* if not significant.

3.7 Acknowledgments

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4.0 HCV NS5A hyperphosphorylation is involved in viral translation modulation

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4.1 Authors' contribution

All the experiment within this chapter were performed by Mangyung Kandangwa. The manuscript was written by Mangyung Kandangwa and edited by Qiang Liu.

4.2 Abstract

Hepatitis C virus (HCV) non-structural (NS) 5A protein is a multifunctional phosphoprotein. NS5A exists as hypo- and hyper-phosphorylated forms and the dynamic transitions between these two states are involved in the functions of NS5A. Hyperphosphorylation of six serine residues within the low complexity sequence I is critical for viral replication and assembly. We previously showed that NS5A downregulates viral translation. In this study, we investigated the role of NS5A hyperphosphorylation in translation modulation. By analyzing the effects of phospho-ablative and phospho-mimetic mutants of the six serine residues on translation, we showed that phosphorylation of S222, S225, S235 is not involved in translation downregulation by NS5A. In contrast, NS5A with alanine mutations at S229 or S238 can no longer downregulate translation, whereas S229D or S238D mutations have no effect. Interestingly, S232D NS5A, but not S232A, abrogates translation downregulation by NS5A. Since dimerization of NS5A plays an important role in its functions, we also studied the effects of phospho-mutants of S229, S232, and S238 on dimerization in a protein-protein interaction assay. We showed that phopho-mimetic S229D or S238D mutations enhances NS5A dimerization, whereas the phospho-ablative mutations of these two residues have no effect. Neither phospho-ablative nor phopho-mimetic mutations of S232 affect dimerization. These results indicate that phosphorylation of NS5A at S229, S232, and S238 is involved in viral translation regulation and NS5A dimerization.

4.3 Introduction

Hepatitis C virus (HCV) is a positive-sense single-stranded RNA virus and belongs to the *Flaviviridae* family (Dustin and Rice, 2007). The genome is 9.6 kb long with one open reading frame encoding a polyprotein of about 3000 amino acids. The polyprotein is co- and post-translationally cleaved by viral and host proteases to yield three structural proteins (core, E1 and E2) and six non-structural proteins (NS2 to NS5B). The structural proteins together make up the virus particle and the non-structural proteins functions in the other stages of the HCV life cycle such as replication and translation. The viral genome also contains untranslated regions (UTRs) at its 5' and 3' ends. These UTR sequences play multiple roles in the viral life cycle (Niepmann et al., 2018).

At present, HCV infection can be effectively treated with direct-acting antivirals (DAAs) (Alazard-Dany et al., 2019; Gitto et al., 2017). DAAs target the non-structural proteins involved in polyprotein processing (NS3/4A protease complex) and genome replication (RNA-dependent RNA polymerase NS5B). There are also DAAs that target NS5A, a viral protein essential for replication. However, how these drugs work to disrupt the functions of NS5A remains obscure.

NS5A is a multifunctional phosphoprotein that is critical for viral replication and viral assembly (Ross-Thriepland et al., 2015). NS5A is also an RNA binding protein (Huang et al., 2005; Hwang et al., 2010). Although it binds to both UTR sequences, NS5A has high binding affinity to the poly(U/UC) track in the 3'UTR (Huang et al., 2005). NS5A consists of an N-terminal amphipathic helix (AH) and three domains (domains I, II, III) separated by low complexity sequences LCS I and LCS II (Fig. 4.1a). Domain I is highly structured while domains II and III are intrinsically disordered. Domain I mediates NS5A dimerization which is involved in RNA binding and viral replication (Lim et al., 2012; Shanmugam et al., 2018). Domain II plays a role in replication through its interaction with cellular protein cyclophilin A (CypA) which stimulates RNA binding. Domain III is involved in virion production and appears nonessential for RNA replication as insertions of foreign sequences in this region have a minimal effect on replication.

It has been known for years that NS5A has two phosphorylated isoforms with apparent molecular weights of 56 kDa and 58 kDa in SDS-PAGE, respectively (Ross-Thriepland et al.,

2015). The phospho-isoforms are generally termed as hypophosphorylated (p56) and hyperphosphorylated (p58) NS5A. NS5A hyperphosphorylation has been shown to play a role in virus replication and assembly. Mass spectrometry, reverse genetics and phospho-proteomics studies have identified a serine rich cluster in LCS I responsible for hyperphosphorylation. In this cluster, eight serine residues are highly conserved and six of them have been identified as phosphorylation sites: S222, S225, S229, S232, S235 and S238. Several serine kinases have been identified that are responsible for NS5A hyperphosphorylation, such as Casein kinase Iα (CKIα) (Ross-Thriepland et al., 2015). Phosphorylation of a serine residue by CKIα is primed by a phosphorylation event at the -3 position with a typical (pS/pT) XXS sequence. The serine cluster of NS5A contains three such motifs for CKIα and sequential phosphorylation cascade events from S229 to S232, then S235 and finally S238 have been demonstrated (Hsu et al., 2018; Quintavalle et al., 2007).

Our previous research showed that NS5A downregulates viral translation dependent upon NS5A binding to the poly(U/UC) sequence in the 3'UTR (Hoffman et al., 2015a; Hoffman et al., 2015b). In this study, we investigated the role of NS5A hyperphosphorylation in translation modulation. Using a genetic approach, we found that hyperphosphorylation of three serine residues is involved in this process. We also showed that NS5A hyperphosphorylation at different sites has various effects on its dimerization.

4.4 Results

4.4.1 NS5A hyperphospho-ablative mutant no longer downregulates viral translation

To study the effect of NS5A hyperphosphorylation on viral translation, we generated phospho-ablative (S6A) and phospho-mimetic (S6D) mutations at the six serine residues of HCV-1b NS5A LCS I (S222, S225, S229, S232, S235, and S238) in the context of NS3-NS5A (Fig. 4.1a) (Neddermann et al., 1999a). To demonstrate NS5A protein expression and phosphorylation status, we performed Western blotting. As shown in Fig. 4.1b, in the context of NS3-NS5A, wild-type NS5A existed as two bands, consistent with hyper- and hypo-phosphorylated forms of NS5A. In contrast, the S6A and S6D NS5A mutants showed a single protein band, corresponding

to the heavier (hyperphosphorylated) or the lighter (hypophosphorylated) species of NS5A, respectively. When plasmids expressing wild-type, S6A, or S6D mutant NS3-NS5A were co-transfected with a replication defective HCV-1b genomic ΔGDD RNA in Huh-7 cells, we found that S6D NS5A downregulated the viral RNA translation to the same extent as wild-type, whereas S6A NS5A did not (Fig. 4.1c). These results indicate that hyperphosphorylation is involved in translation downregulation by NS5A.

4.4.2 Phospho-mutations at S229, S232, and S238 have differential effects on viral translation regulation by NS5A

To map out the role of phosphorylation at each of the six serine residues in translation downregulation by NS5A, these serine residues were mutated to either alanine or aspartic acid, one at a time in the NS3-NS5A construct and their effects on viral translation studied. Huh-7 cells were co-transfected HCV RNA genomic ΔGDD RNA and plasmids expressing NS3-NS5A with single amino acid mutations before viral translation was measured. We found that NS5A with phospho-ablative and phospho-mimetic mutations at S222, S225, or S235 could still downregulate viral translation (Fig. 4.2a and 4.2c), suggesting that phosphorylation at these three serine residues is not involved in translation modulation. In the case of S229 and S238, alanine mutation led to a loss of viral translation downregulation (Fig. 4.2a), whereas aspartic acid mutation at the same sites rescued the translation downregulation by NS5A (Fig. 4.2c), implicating S229 and S238 phosphorylation in translation downregulation by NS5A. In contrast, the S232D mutant did not downregulate translation (Fig. 4.2c), while this function was conserved with alanine mutation (Fig. 4.2a), suggesting that phosphorylation at S232 has a negative effect on translation downregulation by NS5A. Taken together, these results suggest that phosphorylation at different serine residues has various effects on translation downregulation by NS5A. Expression of NS5A proteins was demonstrated by Western blotting (Fig. 4.2b and 4.2d).

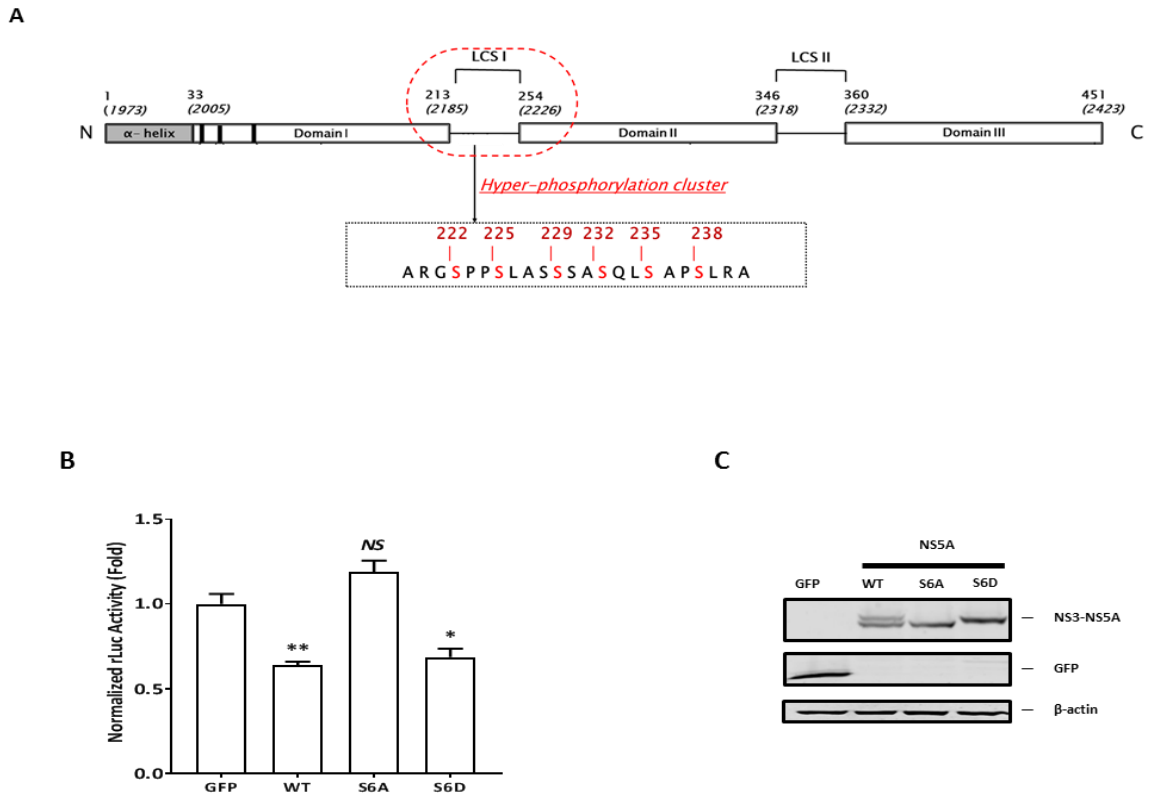

Figure 4. 1: **NS5A with phospho-ablative mutations does not downregulate viral translation.** (A) Schematic diagram of NS5A protein domain structure. The red circle highlights the serine residues involved in NS5A hyperphosphorylation. (B) To demonstrate the status of NS5A phosphorylation, Huh-7 cells were co-transfected with plasmids expressing Myc-tagged GFP, NS3-NS5A, wild-type (WT), phospho-ablative (S6A), or phospho-mimetic (S6D) mutants, together with HCV-1b genomic rLuc translation RNA. Cells were harvested at 24 hr after transfection and Western blotting performed with a Myc-tag antibody. As a protein loading control, the level of β-actin was also determined with a β-actin antibody. The positions of p58 and p56 are indicated. (C) For translation assay, Huh-7 cells co-transfected as in (B) were harvested at 8 hr post-transfection for luciferase assay. The normalized rLuc activity after GFP expression was set to 1. * = $P < 0.05$, ** = $P < 0.01$, *NS* = not significant.

4.4.3 The effect of S238 phosphorylation on viral translation is not regulated by S229 phosphorylation.

Studies have shown that S229 phosphorylation primes phosphorylation cascades which eventually results in the phosphorylation of S238 (Hsu et al., 2018; Quintavalle et al., 2007). Since our data suggested that phosphorylation at both S229 and S238 is involved in translation downregulation by NS5A (Fig. 4.2a and 4.2c), we were interested in studying whether the effect of S238 phosphorylation on viral translation is regulated by the phosphorylation states of S229. We therefore generated two NS3-NS5A constructs carrying double amino acid substitutions S229A-S238D or S229D-S238A and used them in the translation assay. As shown in Fig. 4.2E, the S229A-S238D double mutant showed the same inhibitory effect on translation as the S238D single mutant, whereas S229D-S238A double mutant exhibited the same phenotype as the S238A single mutant. These results suggest that the phosphorylation states of S229 do not affect the role of S238 phosphorylation in translation regulation. Protein expression was demonstrated by Western blotting (Fig. 4.2f).

4.4.4 The effects of phospho-mutations at S229, S232 and S238 on dimerization

NS5A exists as a dimer and dimerization of NS5A is critical for RNA binding and replication (Lim et al., 2012; Shanmugam et al., 2018). Previously, our group has demonstrated that binding of NS5A to the viral RNA is important to downregulate the translation (Hoffman et al., 2015a; Hoffman et al., 2015b). Results so far indicated that phosphorylation of S229, S232, and S238 plays variable roles in translation regulation by NS5A. Hence, we wanted to investigate the effect of NS5A phosphorylation on dimerization and thereby on RNA binding as a potential mechanism for translation modulation. NS5A dimerization was measured by split luciferase complementation assay (SLCA) as previously described (Li and Liu, 2018). For this purpose, we generated plasmids expressing fusion proteins of aa. 1-229 (LN) or aa. 230-311 (LC) of Renilla luciferase with NS5A carrying mutations at S229, S232, or S238 (Fig. 4.3a). Plasmids expressing fusion proteins with wild-type or dimerization-defective C39A-C57G NS5A were also generated as positive and negative controls in the SLCA assay (Lim et al., 2012). As expected,

the wild-type LN-NS5A/LC-NS5A pair resulted in more than two-fold increase in luciferase activity in comparison to vector LN/LC control and the LN-NS5A/LC-NS5A C39A-C57G pair (Fig. 4.3). When S229D or S238D NS5A mutants were used in SLCA, we observed significantly higher luciferase activities than wild-type, whereas the respective alanine mutations had no such an effect (Fig. 4.3b and 4.3c). On the other hand, however, neither S232A nor S232D affected luciferase readings (Fig. 4.3b and 4.3c). These results suggest that phosphorylation at S229, S232, and S238 has various effects on NS5A dimerization.

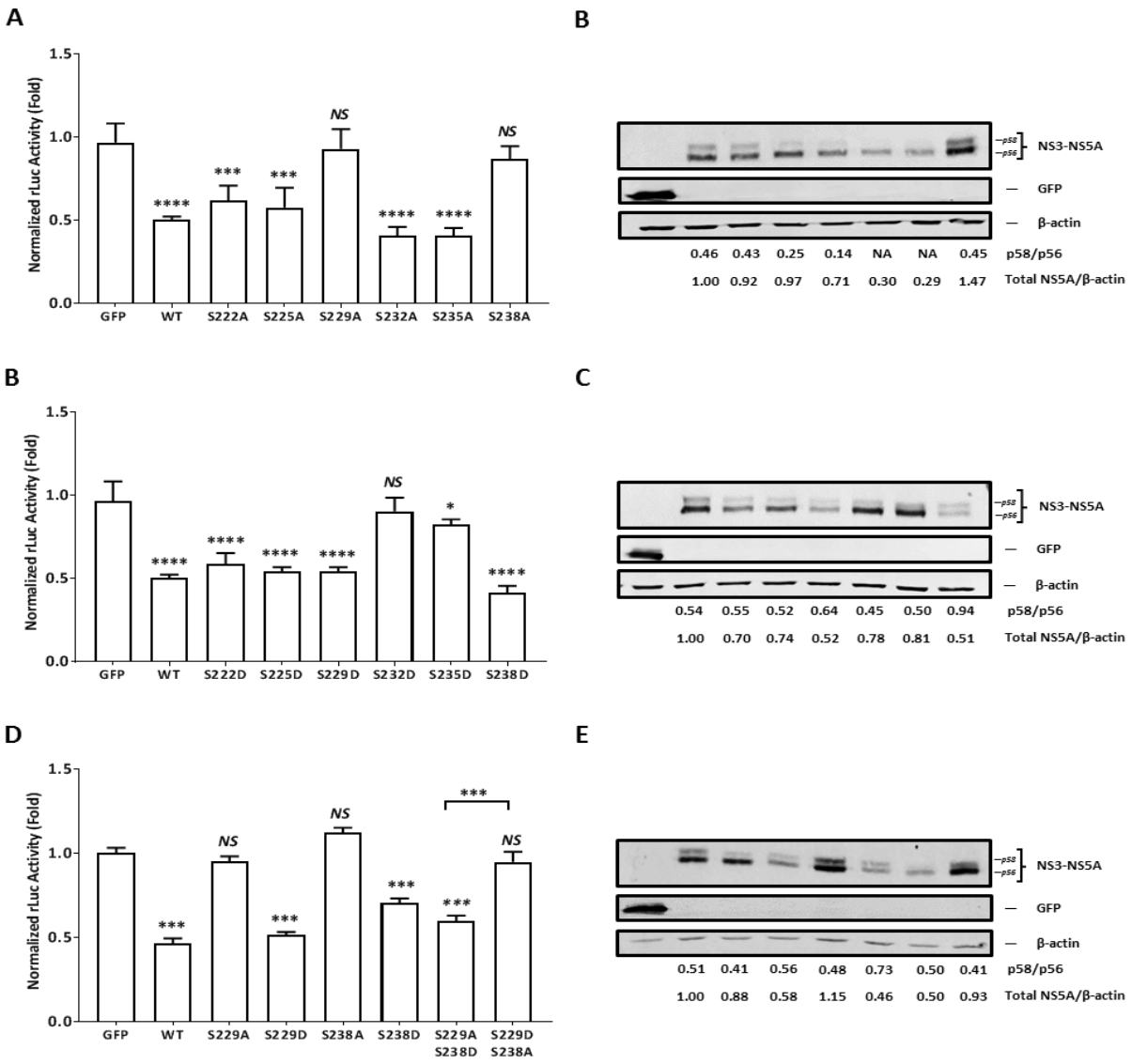

Figure 4. 2: **NS5A phospho-mutants at individual serine residues have different effects on viral translation.** (A, C, and E) Huh-7 cells were co-transfected with plasmids expressing Myc-tagged GFP, NS3-NS5A, wild-type (WT), single phospho-ablative (A), single phospho-mimetic (C), or single and double mutants (E), together with HCV-1b genomic rLuc translation RNA. Cells were harvested 8 hr post-transfection for luciferase assay. The normalized rLuc activity after GFP expression was set to 1. * = $P < 0.05$, *** = $P < 0.001$, **** = $P < 0.0001$, *NS* = not significant. (B, D, and F) Transfected Huh-7 cells were harvested at 24 hr for NS5A protein detection by Western blotting with a Myc-tag antibody. The positions of p58 and p56 are indicated. The level of β-actin was determined with a β-actin antibody to control protein loading. Protein band intensities were analyzed by densitometry and the relative ratios of p58/p56 and total NS5A/β-actin presented underneath the blots.

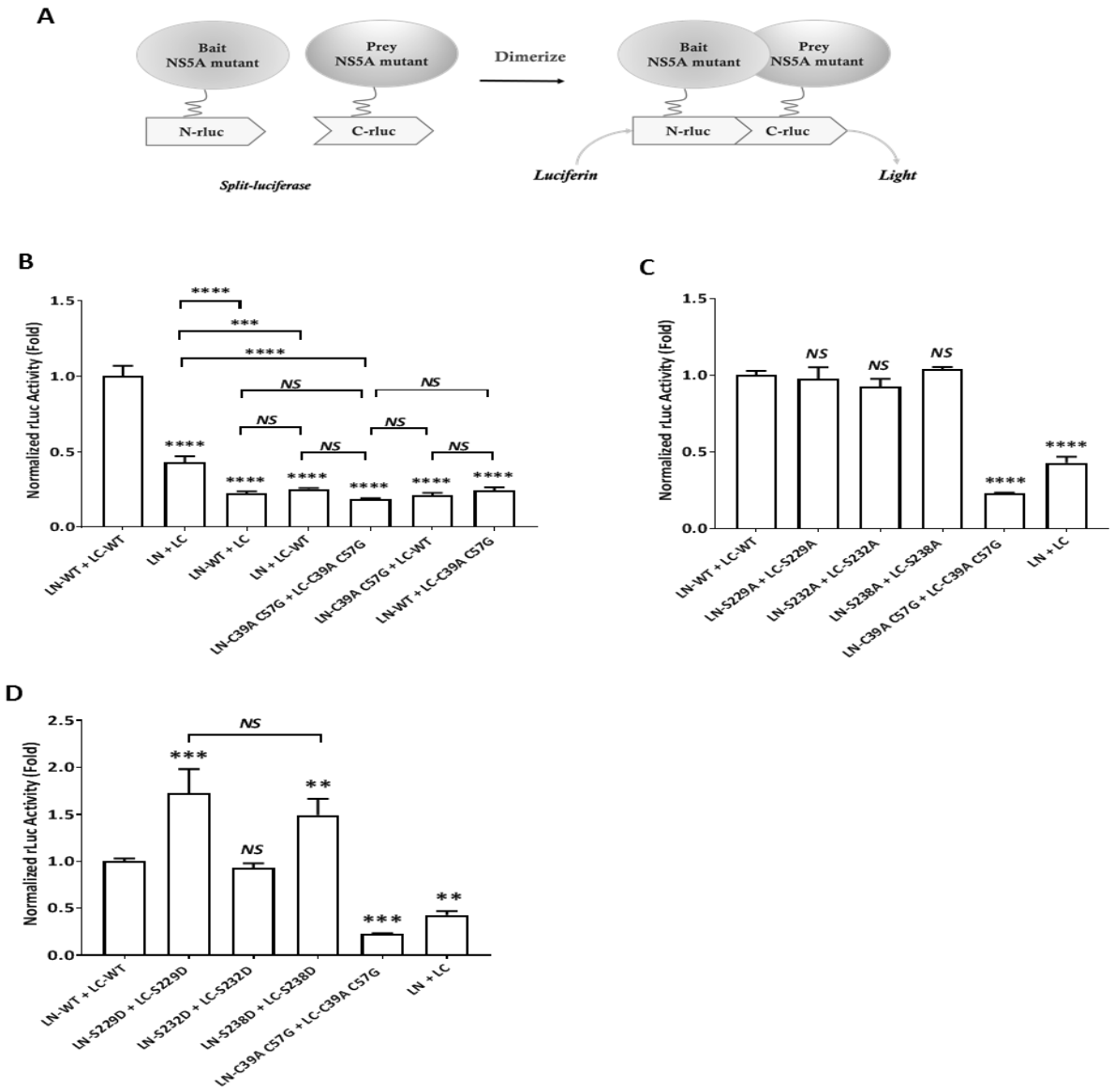

Figure 4. 3: **Phospho-mimetic mutations at S229 and S238 enhances NS5A dimerization.** (A) Principle of split luciferase of complementation assay (SLCA). (B, and C) Huh-7 cells were co-transfected with LN and LC vector pair, or LN-NS5A and LC-NS5A pairs carrying phospho-ablative (B) or phospho-mimetic (C) mutations at S229, S232, or S238 as indicated. LN and LC pairs encoding wild-type or C39A-C57G mutant NS5A were included as controls. Cells were harvested at 24 hr after transfection for luciferase assay. The luciferase activity from LN-NS5A WT + LC-NS5A WT was set to 1. *** = $P < 0.001$, **** = $P < 0.0001$, *NS* = not significant.

4.5 Discussion

HCV NS5A is a phosphoprotein, existing as hypo- and hyper-phosphorylated forms. So far, the role of NS5A hyperphosphorylation in regulation of viral RNA replication and virion assembly has been reported, however, its effects on viral RNA translation and NS5A dimerization have not been addressed. Here, for the first time, we showed that hyperphosphorylation of NS5A is involved in viral translation downregulation and NS5A dimerization.

Numerous studies have shown that the highly conserved six serine residues of NS5A contribute to hyperphosphorylation. Substituting phospho-residues by mutagenesis is an effective tool for understanding the biological relevance of the phosphorylation. Since it has been widely used to study NS5A hyperphosphorylation, we employed this approach in the study. To understand the effect of NS5A hyperphosphorylation on translation, all six serine residues were mutated to either alanines (S6A) or aspartic acids (S6D) to mimic different phosphorylation states. As expected, while both p58 and p56 species can be detected for the wild-type NS5A, S6A NS5A and S6D NS5A exist only as a single protein band, suggesting they represent hypo- or hyper-phosphorylated forms of NS5A (Fig. 4.1). Then these two NS5A phospho-mutants were used in a translation assay using an HCV genomic RNA. While the S6D mutant still downregulates translation as the wild-type NS5A, alanine substitutions (S6A) completely abolished the inhibitory effect on translation (Fig. 4.1). These results strongly suggest that NS5A hyperphosphorylation is involved in translation modulation. At this moment, we do not have a clear understanding on the apparent phenotypical discrepancy between the S6A and S6D mutants. It should be noted, however, that the genomic translation reporter RNA used in the assay expresses wild-type NS5A that is presumably undergoing dynamic phosphorylation-dephosphorylation modifications. Translation measured is the net effect exerted by wild-type NS5A and S6D or S6A mutants. We would like also to point out that this kind of phenotypical discrepancy between phospho-mutants of NS5A is not unprecedented. For instance, an NS5A S222D mutant has been shown to inhibit viral replication, whereas the S222A mutant has no (enhancing) effect (Lemay et al., 2013).

To get insights into the phosphorylation of which of the six serine residues plays a role in translation regulation, we used single phospho-mutants to measure translation. We found three functional categories (Fig. 4.2). S222, S225, and S235 are not involved in translation regulation as both ablative and mimetic mutations have the same inhibitory effects on translation (group 1). S229 and S238 belong to the second group: phospho-ablative mutants can no longer downregulate translation, while the phospho-mimetic mutants can. S235 phospho-mutants have the opposite phenotype to S229 and S238 in group 3. Interestingly, this trend continued when NS5A dimerization was measured using these mutants (Fig. 4.3). Both S229D and S238D, but not S229A and S238A, mutations enhance dimerization. Phospho-mutants of S232 have no effect on dimerization. Taken together, these results suggest that S232 phosphorylation regulates viral translation through a different mechanism from S229 and S235. Further study is warranted.

What are the effects of the phosphorylation at these three serine residues on viral replication? For genotype 1b HCV, the same genotype as in this study, S229A and S229E mutants increase HCV subgenomic RNA replication, whereas S238A and S238E mutants have no effects (Appel et al., 2005). Phospho-ablative S232A mutant, but not phospho-mimetic S232E mutant, enhances viral replication (Appel et al., 2005). Combining these replication data with our translation results, it suggests that phosphorylation at S229 and S238 has quite different effects on replication and translation, whereas the phosphorylation at S232 has a more consistent effect on replication and translation.

In conclusion, we have shown that NS5A phosphorylation at individual serine residues in the LCS I cluster has different effects on translation regulation and NS5A dimerization. These results increase our understanding on the complex functions of NS5A phosphorylation.

4.6 Material and methods

4.6.1 HCV RNA translation reporters and expression plasmids

HCV-1b monocistronic RNA translation renilla luciferase (rLuc) reporter and HCV-1b genomic p7-rLuc2A ΔGDD RNA constructs were described previously (Hoffman et al., 2015a). The plasmids were linearized by *Xba*l (New England Biolabs) and then was *in vitro* transcribed by MEGAscript T7 transcription kit (Ambion). To generate a plasmid expressing HCV-1b NS3-NS5A with a Myc-tag at the C-terminus, the coding sequence was amplifed by PCR using HCV-1b N Neo C-5B as the template and cloned into the pEF vector (Ikeda et al., 2002). Phospho-mutations of NS5A were generated by site-directed mutagenesis. The coding sequence for GFP with an Myc-tag was amplified from pGFP-C1 (Takara Bio USA) and cloned into the pEF vector. Split luciferase complementation assay vectors pLC and pLN, kindly provided by Feng Li (Deng et al., 2011), were used to generate pLC-NS5A and pLN-NS5A plasmids with or without phospho-mutations. Plasmids were confirmed by DNA sequencing.

4.6.2 Cell lines, transfections and luciferase assay

Huh-7 cells were maintained in Dulbecco's modified Eagle's medium (MilliporeSigma) and supplemented with 10% (v/v) FBS (MilliporeSigma) and 1% penicillin-streptomycin and cultured at 37°C and 5% CO_2. The cells were co-transfected with plasmid expressing protein of interest and HCV-1b translation reporter RNA using Jet-PEI transfection reagent (Polyplus-Transfection). At pre-determined time after transfection, cells were lysed with Passive Lysis Buffer and rLuc activity measured using the GloMax 20/20 Luminometer according to the manufacturer's instructions (Promega). The luciferase readings were normalized to total protein concentration which were determined by Bradford protein assay (Bio-Rad).

4.6.3 Western blotting and antibodies

Cell lysates were subjected to 10% or 7.5% SDS-PAGE and transferred onto nitrocellulose membranes. The membranes were blocked with 5% skimmed milk in PBS for 1 hr at room temperature before incubation with a primary antibody overnight at 4°C. After

washing, the membranes were then incubated with the appropriate infrared dye-labelled secondary antibodies for 1 hr at room temperature. The membranes were then washed with PBST (PBS + 0.1% Tween 20) and scanned using Odyssey CLx Imaging System (Li-Cor Biosciences). Myc-tag, and β-actin antibodies were purchased from Cell Signaling Technology. Secondary antibodies IRDye 800 CW goat anti-mouse IgG and IRDye 680 goat anti-rabbit IgG were from Li-Cor Biosciences.

4.6.4 Statistical analysis:

All experiments were done in triplicates and the experimental data analysed using the GraphPad Prism 7. Statistical differences were determined by Student's *t*-test or two-way ANOVA and statistical significance was demonstrated as follows: * if $P < 0.05$, ** if $P < 0.01$, *** if $P < 0.001$, **** if $P < 0.0001$, *NS* if not significant.

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5.0 CONCLUSIONS AND FUTURE WORKS

NS5A is an essential viral protein involved in viral replication and assembly. NS5A's role in translation has not been well understood yet. Thus in our work, we try to shed light on the role NS5A n viral RNA translation.

Our previous work on HCV-1b NS5A showed NS5A to downregulate HCV-1b RNA translation through a mechanism that requires the binding of NS5A to poly(U/UC) region in the viral RNA 3' UTR. All three domains of HCV-1b NS5A were capable of downregulating translation albeit lower full-length wild-type NS5A. HCV has seven major genotypes and whether the role of NS5A on translation is conserved in all genotypes has not been tested yet. In case of replication, NS5A is crucial for both HCV-1b and HCV-2a however, the mutations on NS5A that favored replication of HCV-1b was found to be deleterious for HCV-2a. This further suggest that, although the overall function of NS5A may be conserved in all genotypes but the mechanism of action could be different. Therefore, the first part of the thesis studied the role of HCV-2a NS5A in HCV-2a RNA translation. Our work suggests that HCV-2a NS5A is also involved in translation downregulation of HCV-2a; however, the mechanism is different. In contrast to HCV-1b, HCV-2a NS5A was capable of downregulating translation in the absence of poly(U/UC) region at the viral RNA 3'UTR. In addition, HCV-2a predominantly downregulates translation through its domain I and the effect is independent of the viral 5' and 3' UTR. The actual mechanism through which HCV-2a NS5A downregulates translation is still unclear and further research is required. Some of the future works could include:

1) Study the effect of NS5A domain I residues on replication to understand their significance in other stages of viral life cycle.
2) Study the effect of dimer defective NS5A on translation, to highlight whether RNA binding supported by NS5A dimerization is involved in translation modulation or not.
3) Study the significance of phosphorylation at S146 on translation in terms of full-length wild-type NS5A.
4) Study whether phosphorylation states of HCV-2a NS5A have a role in translation modulation.

NS5A is a multifunctional protein and its different phosphorylation states has been hypothesized to carry out the plethora of its functions. The six serine residues S222, S225, S229, S232, S235 and S238 in the NS5A LCS I serine rich cluster are critical for hyperphosphorylation of NS5A. It has been shown that NS5A hyperphosphorylation can modulate viral replication. The effect of NS5A hyperphosphorylation on translation has not been studied. Thus, the second part of the thesis explored whether HCV-1b NS5A hyperphosphorylation has a role in viral RNA translation modulation. We found that the serine residues can be categorized into three distinct groups based on their effect on translation. Group 1 consist of the residues whose phosphorylation is not involved in translation regulation by NS5A and includes S222, S225 and S235. Group 2 consist of the residues whose phosphorylation is required for translation downregulation by NS5A and includes S229 and S238. Lastly, group 3 consist of serine residues whose phenotype is opposite of group 2 i.e. phosphorylation led to loss of translation downregulation function of NS5A while the function is rescued by blocking phosphorylation through phospho-ablatant mutation. S235 belongs to group 3. In terms of NS5A dimerization, the phospho-mimetic mutants of group 2 residues: S229D and S238D, but not the phosphor-ablative mutants S229A and S238A, enhanced dimerization. While no effect on dimerization was observed for phospho-mutants of S232. Altogether, our results suggest that S232 phosphorylation regulates viral translation through a different mechanism from S229 and S235. The underlying mechanism is still unclear and further studies will be required. Some of the future works could include:

1) Study the role of cellular kinase like polo-like kinase or calmodulin-dependent kinases II (CAMK2) γ and δ on NS5A hyper-phosphorylation mediated translation modulation.
2) Study the effect of NS5A inhibitor on NS5A phosphorylation and viral translation modulation by NS5A.

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